E2F1-INDUCED EXPRESSION OF TRANSACTIVATING AND DOMINANT-NEGATIVE FORMS OF p73 TRANSCRIPTS

A THESIS
SUBMITTED TO DEPARTMANT OF
MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
Ozgur Karakuzu
August 2002

To my family……

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof.Dr.Mehmet Öztürk

I certify that I have read this thesis and that, in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Assoc.Prof.Dr.Ergün Pınarbaşı

I certify that I have read this thesis and that, in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Tamer Yağcı

Approved for the Institute of Engineering and Science.

Prof.Dr.Mehmet Baray

Director of Institute of Engineering and Science

ABSTRACT

E2F1-INDUCED EXPRESSION OF TRANSACTIVATING AND DOMINANT-NEGATIVE FORMS OF p73 TRANSCRIPTS

Ozgur KARAKUZU
M.S. in Molecular Biology and Genetics
Supervisor: Prof. Dr. Mehmet OZTURK
August 2002, 102 pages.

Cell cycle, one of the most important life processes, is controlled by a regulated balance between proliferative and anti-proliferative signals. Dysregulation of these signals leads to tumor development. Retinoblastoma (Rb) gene is the principle regulator of the cell cycle. Rb was identified initially as a gene deleted in a rare form of early child eye tumor, called retinoblastoma, and it was later shown to be a tumor-suppressor. Cellular functions of Rb are inactivated in many cancer types, either directly by Rb gene mutation, or indirectly by inactivation of the pRb protein that is mediated by different viral oncogenes. pRB exists in non-proliferating (quiescent) cells as a complex with E2F transcription factors. Upon phosphorylation of pRb by cyclin-dependent kinases, E2Fs are released and can transactivate their target genes. E2F1, the first E2F to be identified, activates mostly proliferative genes, but also anti-proliferative genes such as $p14^{ARF}$ that acts as an inducer of p53 stabilization. In turn, p53 induces either cell cycle arrest or programmed cell death (apoptosis). Recently, it was reported that E2F1 also induces p53-independent apoptosis by transactivating the expression of the p53 homologue p73 gene. However, p73 encodes not only apoptosis-inducing transciptionally active(TA)-p73, but also dominant negative (DN)-p73 transcript forms which antagonize TA-p73. Our aim was to investigate whether the E2F1 activates the expression of TA-p73, DN-p73 or both. We over-expressed E2F1 and E2F4 in different human cell lines, by transient transfection using appropriate expression vectors, and analyzed p73 transcript levels by semi-quantitative RT-PCR. We demonstrate that, in different cell lines, E2F1 induced the expression of not only TA-p73, but also its two dominant-negative forms, namely p73Deltaexon2 and DN-

p73. Time course studies indicated that TA-p73 and p73DeltaExon2 forms are induced initially, and DN-p73 induction is delayed about 4 hours. Induced expression of dominant-negative forms, in addition to transcriptionally active p73 transcripts by E2F1 may explain how some cancer cells are able to tolerate p73 activation in response to oncogenes such as E2F1.

ÖZET

EF21 TRANSKRİPSİYON FAKTÖRÜ TARAFINDAN p73'ÜN AKTİF VE DOMİNANT-NEGATİF TRANSKRİPT FORMLARININ ENDÜKLENİŞİ

Özgür Karakuzu
Moleküler Biyoloji ve Genetik Yüksek Lisans
Tez Yöneticisi: Prof. Dr. Mehmet Öztürk
Ağustos 2002, 102 Sayfa

Yaşam için en önemli işlemler arasında yer alan hücre döngüsü, çoğaltıcı ve çoğalmayı engelleyici uyarıların karşılıklı olarak dengelenmesi yolu ile denetlenir. Bu uyarılardaki düzensizlik tümör gelişimine yolaçmaktadır. Retinoblastoma (Rb) geni hücre döngüsünün ana düzenleyicidir. Bir çocukluk çağı göz tümörü olan retinoblastomalarda delesyona uğrayan bir gen olaran ortaya çıkarılan Rb geninin, tümör baskılayıcı bir gen olduğu sonradan belirlenmiştir. Rb geninin hücre işlevleri, ya doğrudan mutasyonla, ya da dolaylı olarak pRb proteininin virüs onkogenlerince etkisiz hale getirilmesiyle, bir çok tümör türünde kaybolmaktadır. pRB proteini, çoğalmayan (dingin) hücrelerde, E2F transkipsiyon faktörleri ile birlikte bir kompleks halinde bulunur. pRb'nin siklin-bağımlı kinazlar tarafından fosforlanması üzerine, E2F'ler serbest kalır ve böylece hedef genlerini aktif hale getirebilir. İlk belirlenen E2F olan E2F1, sıklıkla çoğaltıcı genleri uyarmakla birlikte, çoğalmayı engelleyen genleri de, örneğin p53 proteinini stabilize eden $p14^{ARF}$ genini de uyarabilir. Bunun sonucu olarak, p53 hücre döngüsünü durdurur veya programlı hücre ölümüne (apoptoz) neden olur. Daha yakın bir zamanda, E2F1'in p53'den bağımsız olarak ve bir p53 homoloğu olan p73 genini uyarma yolu ile de apoptoza yol açtığı bildirildi. Ancak, p73, sadece apoptoz etkisi olan aktif (TA) p73 değil, aynı zamanda TA-p73 antagonisti olan dominant negatif (DN) p73 transcript formları da kodlayabilmektedir. Bu çalışmanın amacı E2F1'ın TA-p73'ü mü, DN-p73'ü mü, yoksa her iki form birlikte mi uyardığını belirlemekti. Bu amaç doğrultusunda, ekspresyon vektörleri ile geçici transfeksiyon yöntemini uygulayarak, çeşitli insan hücre dizilerinde E2F1ve E2F4'ün ifade edilmesini

sağladık ve yarı-kantitatif RT-PCR'la p73 transkript düzeylerini inceledik. Bu çalışmalarımız, çeşitli insan hücrelerinde, E2F1'in sadece TA-p73'ü değil, ayrıca bu molekülün dominant-negatif formlarını, yani p73Deltaexon2 ve DN-p73'ü de uyardığını gösterdi. Zaman akışlı incelemelerle, önce TA-p73 ve p73DeltaExon2 formlarının uyarıldığını, DN-p73 uyarımının ise yaklaşık 4 saat sonra gerçekleştiğini gözlemledik. E2F1 tarafından p73'ün TA formuna ek olarak dominant-negatif formlarının da uyarılması, bazı kanser hücrelerinin E2F1 gibi onkogenlere yanıt olarak geliştirilen p73 aktivasyonunu nasıl tolere edebildiklerini açıklayabilir.

ACKNOWLADGEMENT

I would like to thank to

My family for their support all my life,

Dr. Mehmet Öztürk, for his supervision of my master thesis and his patience,

Dr. Ergün Pınarbaşı, for his supervison in my thesis, helps in my personal life and being refree in my thesis defense,

Dr. Tamer Yağcı, for being refree in my thesis defense,

Emre and Berna Sayan's, for their advisory and helps in my thesis, their friendship and patience against me,

Tolga Turan, Tuba Gülbağcı, Ebru Demir, Banu Sürücü, Ahmet Vakkasoğlu, Tahir Malas, for their companianship and helps during my undergraduate and graduate studies,

Füsun Elvan, for her patience and speed in supplying our laboratory materials,

Tülay Arayıcı, for her intensive help in sequencing my DNA samples,

And to all MBG memebers for their helps and friendship during my studies here.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Bp	Base pair
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitor
DNA	Deoxyribonucleic acid
DN-p73	Dominant negative p73
dNTP	Deoxyribonucleotide
Ds	Double stranded
EDTA	Diaminoethane tetra-acetic acid
GFP	Green fluorescence protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
kDA	Kilo Dalton
LB	Luria-Bertani medium
LOH	Loss of heterozygosity
MEF	Mouse embryonic fibroblast
OD	Optical density
P73-Δexon2	p73 delta exon2 spliced form
PAGE	Polyacryalmide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
Rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
TA-p73	Transactivating p73
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane

CHAPTER 1

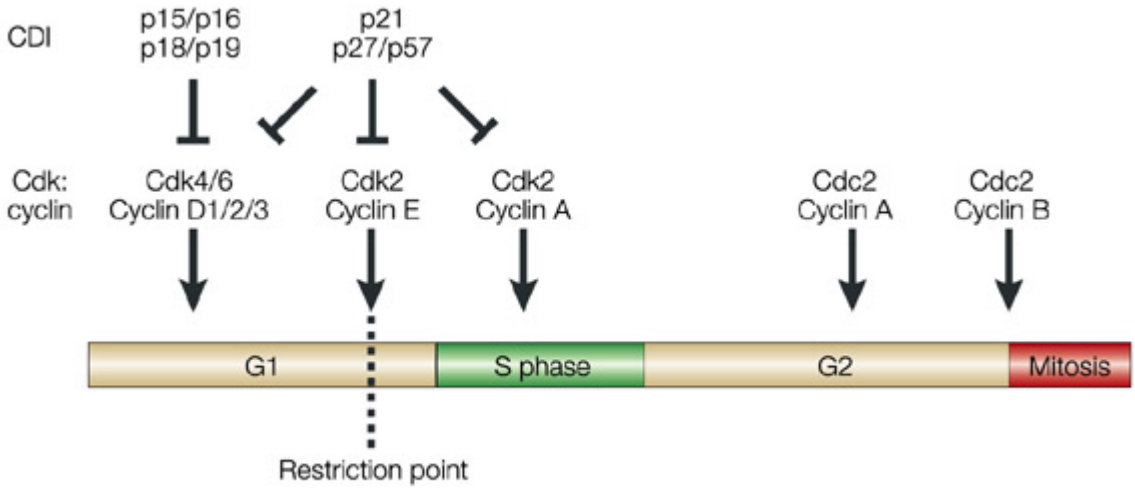
INTRODUCTION

1.1 pRb/E2F PATHWAY

In physiological conditions, mammalian cells are found in different stages such as quiescence (non-proliferation), proliferation and senescence. Cell proliferation is governed by the cell cycle machinery. Cell cycle is regulated strictly, and its dysregulation results in tumorigenesis. Cell cycle is controlled by a finely tuned balance between proliferative and anti-proliferative signals. Cell cycle is under the control of a family of protein kinases called cyclin-dependent kinases (CDKs), activation of which is in a sequential manner during the cell cycle (Sherr and Roberts, 1999). In order to be active, CDKs have to associate with a group of activating proteins called cyclins. Actually there is always a ubiquitous expression and pool of inactive CDKs in cells, but they need to associate with cyclins to be active. Cell cycle is regulated by cellular levels of cyclins. At different stages of cell cycle different couples of CDKs and cyclins take role. For the entry into cell cycle, the step is the activation of CDK4 and CDK6 in association with cyclin D. Activation of cyclin D/CDK4-6 complex triggers the subsequent activation of CDK2, which then associates with cyclin E. Following set of cyclin/kinase complex is the cyclin A/CDK2. Near to the end of replication phase cyclin B/CDK1 (mitosis promoting factor) is activated and mitosis starts. There are many pathways interfering and affecting regulation of cell cycle. Dysregulation in the activities of cyclins and CDKs may lead to cancer development, as it was shown by activating mutation of CDK4 and amplification of cyclin D gene in some cancers (reviewed by Ortega et al. 2002).

The activities of cyclin/CDK complexes are regulated by cyclin dependent kinase inhibitors (CKIs) including INK4 and Cip/Kip family of proteins. $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ are the members of INK4 family and their principle targets are CDK4 and CDK6, which induces the G1-S transition of cell cycle. The Cip/Kip family members, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ seem to have dual roles. They negatively regulate cyclin E-cyclin A/CDK2 complexes, whereas they have activating effect on cyclin D/CDK4-6. Having dual roles for cell cycle, Cip/Kip family proteins are not expected to have inactivating mutations. However for INK4 family of proteins, there are cases of mutations and epigenetic changes (*de novo* methylation) yielding in abnormal cell proliferation

and tumor formation. Figure 1 is a presentation of different regulators of cell cycle.



Jeffrey M. Trimarchi and Jacqueline A. Lees, 2002

Figure 1: Regulation of Cell cycle by proliferative and anti-proliferative signals

The most important substrate of CDKs is the retinoblastoma protein (pRb). The retinoblastoma gene (*Rb*) encodes a 928-amino acid phosphoprotein, which arrests cells in the G_1 phase (Weinberg 1995). pRb is sequentially phosphorylated and dephosphorylated during the cell cycle. The hyperphosphorylated (inactive) form predominates in proliferating cells, whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells (Chen et al. 1989).

Being the first tumor suppressor identified, Rb was cloned as the cause of a rare eye tumor (retinoblastoma) seen in children (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987) Intensive studies about it showed that it is an important actor in cellular regulation. Its tumor suppressor activity was demonstrated by introduction of the wild type Rb into Rb-deficient tumor cells, which led to blocking of malignant phenotypes (Huang et al. 1988). Most striking evidence about its tumor suppressor activity was that mutations of Rb were not just seen in retinoblastoma, but also in many other cancers such as osteosarcoma, small cell lung cancer, prostate cancer, and breast cancer (Friend et al. 1986; Fung et al. 1987; Harbour et al. 1988; Lee et al. 1988; T'Ang et al. 1988; Bookstein et al.

1990). The children with hereditary retinoblastoma have ≥30-fold increased risk of developing other kind of tumors in their lives (Eng et al. 1993; Moll et al. 1997).

In addition to mutations constitutive inactivation of pRb protein occurs by hyperphosphorylation or by binding of some viral oncoproteins such as adenovirus E1A, SV40 large tumor antigen, and human papillomavirus (HPV) E7 (Sherr 1996; DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989).

1.1.1 STRUCTURE OF pRb

Domains A and B are the most important domains of pRb. These domains are highly conserved among many species from human to plants, indicating their importance. Two domains interact with each other forming a central pocket (Chow and Dean 1996; Lee et al. 1998). The identified germline retinoblastoma mutations lead to the disruption of the pocket region (Qin et al. 1992, Horowitz et al. 1990). Viral oncoproteins HPV-16 E7, adenovirus E1A, and SV-40 large T antigen containing LXCXE motif, were shown to bind pRb at the pocket with domain B in a domain A dependent manner (Whyte et al. 1988; Dyson et al. 1989; Ludlow et al. 1989; Lee et al. 1998, Kim and Cho 1997). There are other endogenous proteins binding to pRb having LXCXE-like sequence such as histone deacetylase HDAC1, HDAC2, the ATPase, and BRG1 of the SWI/SNF nucleosome remodeling complex (Dunaief et al. 1994; Brehm et al. 1998; Luo et al. 1998; Magnaghi et al. 1998).

E2Fs, lacking the LXCXE motif, bind pRb at a distinct site involving points of contact in both the pocket and in the C-terminal region (Huang et al. 1992; Lee et al. 1998).

Another region at the C-terminal of pRb is essential for binding of c-Abl tyrosine kinase and MDM2. This site is distinct from the E2F site in the carboxy-terminal region (Welch and Wang 1993; Xiao et al. 1995). pRb inhibits c-Abl when bound and hyperphosphorylation of pRb releases c-Abl (Welch and Wang 1993; Whitaker et al. 1998).

There is not yet clear information about the role of the interaction between pRb and MDM2. In addition to the data showing that MDM2 inhibits pRb, there are also recent indications that pRb may be inhibiting the anti-apoptotic effects of MDM2 by forming a trimeric complex with MDM2 and p53 (Hsieh et al. 1999).

The consensus phosphorylation sites of pRb, which are important for regulation by CDKs, are mostly present in its amino-terminal region. There are also several proteins known to be interacting with amino-terminal such as MCM7 (a replication licensing factor), a novel G_2/M cycle-regulated kinase , and some other proteins with unknown functions (Sterner et al. 1998; Sterner et al. 1995; Durfee et al. 1994).

1.1.2 pRb IN REGULATION OF E2F TRANSCRIPTION FACTORS

Interaction between E2Fs and pRb is the mostly studied part of pRb function. At least two mechanisms were suggested for the repression of E2Fs by pRb. First, binding of pRb to E2Fs, can block its activity to activate transcription (Flemington et al. 1993; Helin et al. 1993). Second, the pRb may form a repressor complex at promoters and can actively repress the transcription of E2Fs (Bremner et al. 1995; Sellers et al. 1995; Weintraub et al. 1995). First mechanism involves the physical block of E2F by pRb binding within the transactivation domain of E2F. However second mechanism was proposed to be mediated by HDACs which were recruited by pRb. HDACs recruited to the promoter region where pRb and E2F are complexed, may block the access of transcription factors to the promoter by remodeling the chromatin structure (Kingston and Narlikar 1999; Kornberg and Lorch 1999; Wolffe and Hayes 1999). On the other hand E2F1 was shown to interact with the histone acetyl transferases p300/CBP and p/CAF (Trouche et al. 1996). Acetylation of E2F1 increases its affinity to DNA (Martinez-Balbas et al. 2000). Recruitment of HDACs by pRb may have role in negation of the acetyltransferase activity of the HATs recruited by E2F1 and acetylation of E2F1 (Harbour JW and Dean DJ, 2000).

Another class of chromatin remodeling complexes is the one dependent on ATP hydrolysis and these complexes influence the binding of transcription factors to the promoters by positioning the nucleosomes (Tyler and Kadonaga

1999; Schnitzler et al. 1998; Lorch et al. 1999). The human homologs of yeast SWI2/SNF2 were characterized as BRG1 and BRM and they were found to be interacting with pRb (Dunaief et al. 1994; Singh et al. 1995). Each multi-subunit of SWI/SNF complexes contains an ATP-ase subunit. Several studies suggested that these complexes might have role in transcriptional activation of some promoters by recruiting some activators and HATs (Cosma et al. 1999; Tyler and Kadonaga 1999). On the other hand it was also shown that mutant *SWI2/SNF2* activated more genes than it repressed, which might be due to dual functions of the complex as an activator or repressor (Holstege et al. 1998).

Simultaneous binding of BRM and E2F proteins to pRb, provides another possibility, in which a SWI/SNF-pRb-E2F complex can form on E2F binding sites of promoters (Trouche et al. 1997). Over-expression of BRG1 in BRG1 and BRM deficient cells caused cell cycle arrest in a pRb dependent manner. Furthermore dominant negative forms of BRG1 and BRM blocked the growth suppression by pRb (Dunaief et al. 1994; Strobeck et al. 2000). A recent document showed that both HDAC and SWI/SNF complexes could be recruited to a single complex by pRb.

Singh *et al* suggested a role for pRb in activation of transcription on some glucocorticoid receptor promoters. It is possible that pRb-SWI/SNF complex recruited to the glucocorticoid receptor where HAT activity is dominant, may be responsible for the transactivation role (Singh et al. 1995). In a study MyoD was shown to require pRb for transactivation and induce myogenic differentiation. This effect was thought to be independent of E2Fs (Gu et al. 1993; Sellers et al. 1998).

HDAC independent mechanisms repressing transcription actively were suggested for pRb, possibly involving co-repressors such as CtIP, RBP1, and HBP1 (Luo et al. 1998; Meloni et al. 1999; Yee et al. 1998; Lai et al. 1999a). CtIP inhibits CtBP which is an E1A binding protein (Schaeper et al. 1998). pRb pocket domain was the binding region for CtIP. CtIP had an intrinsic repressor activity, which required a motif mediating interaction with CtBP (Meloni et al. 1999). RBP1 being another pocket binding protein, was shown to be an inhibitor of E2F transactivation and suppressor of cell growth when exogenously expressed (Lai et

al. 1999b). RBP1 is known to recruit HDAC with one of its two repression domains and the HDAC-RBP1-pRb complex might be suppressing transactivation at E2F promoters (Lai et al. 1999a).

1.1.3 REGULATION OF pRb BY PHOSPHORYLATION

Sixteen potential phosphorylation sites for pRb were identified. Such a high number of sites provide the protein to oscillate between hyperphosphorylated to hypophosphorylated and unphosphorylated states. Three different kinase complexes were found to be phosphorylating pRb during cell cycle in a sequential manner. Cyclin D-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2 phosphorylate pRb at early G1, near the end of G1 and during S-phase respectively (Sherr et al 1996) **(Figure 1**). The high number of sites and their phosphorylation status appear to have different consequences for the functions of pRb. For instance, different sets of phosphorylations regulate binding of E2F, LXCXE proteins and c-Abl to pRb (Knudsen and Wang 1996, 1997). First phosphorylation by CDK4/6 makes pRb hypophosphorylated and active (inhibiting E2F). Additional phosphorylation by CDK4/6 leads to hyperphosphorylated and inactive form of pRb in later G1 (Ezhevsky et al. 1997). For a successive phosphorylation both cyclin D-CDK4/6 and cyclin E-CDK2 complexes were found to be required (Lundberg and Weinberg 1998). A mechanism suggested by Harbour *et al* states that cyclin D-CDK4/6 appears to phosphorylate specific sites in the carboxy-terminal region of pRb. An intra-molecular interaction between C-terminal region and a lysine patch, which is positively charged around the LXCXE-binding site in domain B, is triggered. HDAC is removed from the pocket as a result of this interaction. pRb is then unable to repress cyclin E gene (*CCNE*). Cyclin E is expressed as a result of disruption of pRb-HDAC complex by cyclin D-CDK4/6 (Harbour et al. 1999; Zhang et al. 2000). Overexpressed cyclin E was shown to be enough to override Rb-mediated G1 arrest (Zhang et al. 2000).

Interestingly phosphorylation of pRb by cyclin D-CDK4/6 was not only shown to remove HDAC but also recruit cyclin E-CDK2 to the pocket (Adams et al. 1999; Harbour et al. 1999). Cyclin E-CDK2 then facilitated phosphorylation of Ser 567, which was buried within the domain A-domain B interface and not

accessible before phosphorylation by cyclin D-CDK4/6 (Harbour et al. 1999). Phosphorylation of Ser 567 caused release of E2F1 from the pRb. Further data showed that Ser567 was the only phosphorylation site being the target of most missense mutations naturally occurring in tumors (Templeton et al. 1991).

1.1.4 OTHER POCKET PROTEINS

In addition to pRb there are two more pocket proteins identified homologous to pRb, called p107 and p130. Actually the spacer region between two domains A and B of pRb is not conserved in p107 and p130 or among species. This region is only conserved between p107 and p130 and has a p21-like sequence. Spacer region was found to be important for inhibition of the cyclin E-cyclin A/CDK2 complexes, and growth suppression by P107 (Ewen et al. 1992; Zhu et al. 1995b; Adams et al. 1996; Lacy and Whyte 1997). Both homologs were able to bind viral oncogenes, inhibit E2F-responsive promoters, recruit HDAC, actively repress transcription and arrest the growth of cells when over-expressed as pRb (Zamanian and La 1993; Ferreira et al. 1998; Bremner et al. 1995; Starostik et al. 1996; Zhu et al. 1993; Claudio et al. 1994). There were also differences between pocket proteins and their target E2Fs during cell growth and terminal differentiation. pRb was able to bind E2F1-E2F4, however p107 and p130 were able to bind to E2F4 and E2F5. Furthermore it was found that during quiescence and differentiation of muscle cells the abundant pocket protein-E2F complex was the p130-E2F4 (Hijmans et al. 1995; Sardet et al. 1995; Nevins 1998). pRb-E2F complexes were replaced with p130-E2F complexes in myotubes in order to maintain inhibition DNA synthesis (Corbeil et al. 1995; Kiess et al. 1995; Shin et al. 1995). It was suggested by genetically modified animal experiments that three pocket proteins had overlapping and distinct functions. Retinal tumors were detected frequently in chimeric both $Rb^{-/-}$ and $p107^{-/-}$ mice but not seen in chimeric $Rb^{-/-}$ mice. A parallel result was development of $Rb^{-/-}$ mice normally, whereas homozygous loss of p107 in addition to Rb resulted in growth retardation and early mortality (Robanus-Maandag et al. 199; Jacks et al. 1992; Lee et al. 1996). Homozygous deletion of p107 together with homozygous loss of Rb resulted in lethality two day earlier than only homozygous Rb deletion

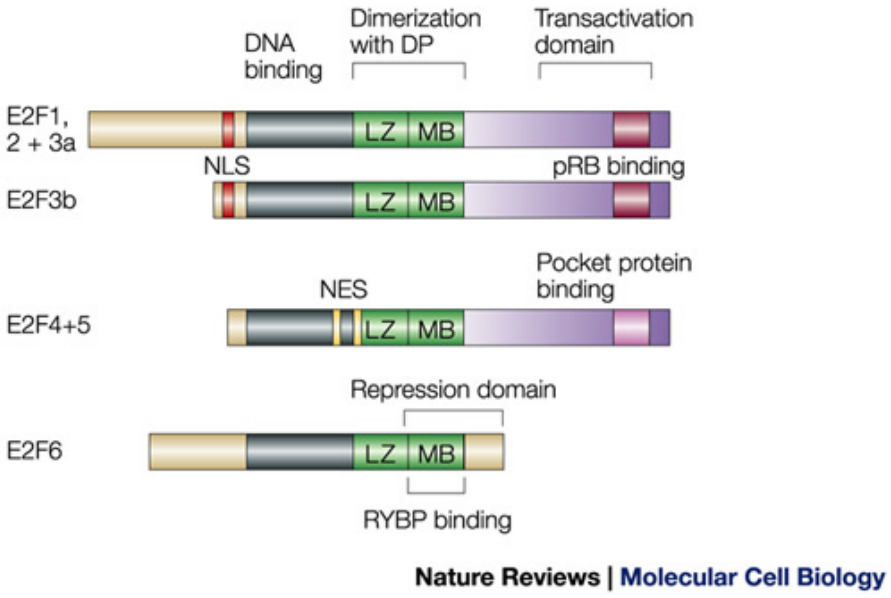
did in mid-gestation (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Lee et al. 1996).

Arrest of cell growth by p16 was known to depend on only Rb, however p16 failed to arrest MEFs which were $Rb^{+/+}$, $p107^{-/-}$, and $p130^{-/-}$. This means in addition to pRb, p107 and p130 are required for p16 mediated growth arrest. Explanation of this situation might be the binding of p107 and p130 to cyclin E/CDK2 and cyclin A/CDK2, and titrating CDK2 activity down to a level that cell cycle arrest by pRb is efficiently done (Hannon et al. 1993; Zhu et al. 1995b)

1.1.5 E2F TRANSCRIPTION FACTORS

There are eight different transcription factors identified to have E2F activity. Due to structural and functional differences made they are divided into two groups as E2F s and DPs. (E2F1-E2F6) and (DP1 and DP2) are the members of two groups respectively (Dyson et al 1998; Helin et al 1998). Any combinations of E2F-DP hetero-dimers possible were identified *in vivo* (Bandara et al 1993; Helin et al 1998; Krek et al 1993; Wu et al 1995; Trimarchi et al 1998). TTTCCCGC was the consensus sequence preferentially recognized by all E2F-DP complexes.

Different transcriptional responses to different E2F–DP dimers depend on the identity of the E2F moiety and other proteins associated with the complex. E2Fs are also divided into three in themselves; activators, repressors and E2F6 (being the last group and its only member).



M. Trimarchi and Jacqueline A. Lees, 2002

Figure 2: Structures of E2F family of proteins

1.1.5.1 The 'activating' E2Fs

These are the potent transcriptional activators and include the E2F1, E2F2 and E2F3. E2F1-DP complex is a potent activator of E2F-responsive promoters, and E2F2 and E2F3 (highly homologous to E2F1) have similar transactivation properties (Bandara et al 1993; Helin et al 1998; Krek et al 1993; Ivey-Hoyle et al 1993; Lees et al 1993) Activating E2Fs may have repressive effects on promoters by recruiting pRb, but over-expression assays and mutant mouse models suggested roles for them in activation of genes essential for cell proliferation and apoptosis.

1.1.5.1.1 Triggering S-phase entry and apoptosis

Any of the activator E2F triggers entry of quiescent cells into cell cycle, with a DNA-binding and transactivation dependent manner when over-expressed (Johnson et al 1993; Qin et al 1994; Lukas et al 1996). In some situations, they

were able to overcome TGF-β, and CKI mediated growth arrest signals (DeGregori et al 1995; Schwarz et al 1995; Mann et al 1995). Transformation of some primary cells was also induced by activator E2Fs (Johnson et al 1995; Shan et al 1994; Singh et al 1994; Xu et al 1995). Blocking of active E2F3 with E2F3 antibodies resulted in cell cycle arrest in primary cells. Moreover, in MEFs $E2F3^{-/-}$ almost all E2F-responsive genes failed to be activated in response to mitogens and the rate of proliferation of primary and transformed cells decreased (Leone et al 1998; Humbert et al 2000). Inactivation of all three E2Fs completely blocked the cell proliferation, suggesting overlapping functions for three E2Fs in proliferation.

On the other hand it was shown that deregulation of E2F1 was found to be inducing apoptosis in both p53-dependent and independent ways (Dyson et al 1998). For p53 dependent manner, $p19^{ARF}$ was thought to be the mediator of the effect. E2F1 was found to be transcriptionally activating $p19^{ARF}$ and $p19^{ARF}$ was known to bind and block MDM2. The free and stabilized p53 then induced apoptosis (DeFregori et al 1997; Bates et al 1998). Alternative p53-independent pathways might be through transactivation of another p53 family or a non-transcriptional mechanism involving TNFR-associated survival factors (Phillips et al 1999; Irwin et al 2000; Lissy et al 2000; Stiewe et al 2000). However there are contradicting data about the potential of three activator E2Fs to induce apoptosis. First group of data suggested that only E2F1 had apoptosis inducing potential. It was shown that MEFs deficient of E2F1 were resistant to c-myc induced apoptosis, but not the ones deficient of E2F2 or E2F3 (DeFregori et al 1997; Lissy et al 2000; Kowalik et al 1998; Leone et al 2001) . On the other hand three of the E2Fs had the similar potential to activate apoptosis in another study. Furthermore it was also shown that neither $E2F1^{-/-}$ nor $E2F3^{-/-}$ MEFs showed a significant difference in apoptotic response than wild types did in response to different apoptotic stimuli. (Vigo et al 1999)

1.1.5.1.2 Regulation of "activator E2Fs"

There is a specific regulation of activating E2Fs by pRb but not by p107 or p130 in normal cells (Lees et al 1993). Upon phosphorylation of pRb in late G1, E2Fs were released and this release correlated with the activation of E2F-

responsive genes. Both over-expression of E2Fs and the functional inactivation of pRb induced the same phenotype in tumors and embryonic tissues (Mulligan et al 1998). Mutation of E2F1 or E2F3 suppressed all these phenotypes including deregulated proliferation and apoptosis (Tsai et al 1998; Ziebold et al 2001).

1.1.5.1.3 Role in normal development

Although activating E2Fs had similar effects on proliferation and apoptosis, deficiency of E2F1 and E2F3 in mice showed completely different developmental phenotypes. There was a high number of *E2F3*$^{-/-}$ mice died *in utero*, and prematurely of congestive heart failure. On the other hand E2F deficient mice were viable and fertile with a number of tissue-specific abnormalities such as an excess of T cells, the development of testicular atrophy between 9 and 12 months of age and development of many kinds of tumors between 8 and 18 months of age (Jeffrey M. Trimarchi and Jacqueline A. Lees, 2002).

Recent studies showed that E2F1 was stabilized as a result of phosphorylations by ATM and ATR. Chemotherapic agents also increased E2F1 protein levels. Further studies indicated that E2F1 might also be involved in the DNA-damage-response pathway (Meng et al 1999, Lin et al 2001) NBS1 and MRE Recombination/repair complex was shown to associate with E2F1 (Maser RS et al 2001). These data suggest further roles for E2F1 and maybe for other members of E2F family in DNA repair mechanisms.

The collaborative effects of E2F1 and E2F2 were shown by Zhu and colleagues in the regulation of haematopoietic cell proliferation, differentiation and tumor suppression (Zhu JW 2001). The developmental defects seen in individual E2F1$^{-/-}$ or E2F3$^{-/-}$ mice deepened in mice defective of both E2F1 and E2F3 (Wu, L. et al 2001) As a conclusion activator E2Fs seem to have overlapping functions in proliferation, induction of apoptosis and development. The important difference seems to be their tumor suppressive effects. It was shown that ***E2F3*** mutation did not result any increase in tumor formation alone or together with E2F1 mutation, as a demonstration of tumor repressive effects of only E2F1 and E2F2 but not E2F3 (Trimarchi & Lees 2002).

1.1.5.2 The 'repressive' E2Fs

The two member of this group E2F4 and E2F5 were called ‘repressive E2Fs’, because E2F-responsive genes were actively repressed by the pocket proteins recruited by these E2Fs. They were found to be associate with both p107 and p130 (Dyson, N. et al 1993; Beijersbergen, R. L. et al 1994; Hijmans, E. M et al 1995; Vairo, G et al 1995) Regulation of repressive E2Fs was shown to be different from activating E2Fs. First of all, the cell cycle stages, at which these factors were detected, differ from each other. Repressive E2Fs were mostly detected at G_0, but activating E2Fs were abundant in actively dividing cells (Ikeda, M. A et al 1996; Moberg, K et al 1996) Second difference was the pocket proteins they bound *in vivo* (Dyson et al 1993; Beijersbergen et al 1994; Hijmans et al 1995; Vairo et al 1995; Ikeda et al 1996; Moberg et al 1996; Lees et al 1993). While pRb was the regulator of activating E2Fs, E2F5 was regulated by p130 and E2F4 was regulated by both pRb and p130. Third level of regulation depends on the cellular localization of the E2Fs. An interesting structural difference between activating and repressive E2Fs is the presence of a NLS (nuclear localization signal) in activating E2Fs and a NES (nuclear export signal) in repressive E2Fs. NLS caused activating E2Fs to be constitutively nuclear. On the other hand repressive E2Fs were kept in the cytoplasm if not associated with pocket proteins. Once they bound to a pocket protein they were taken into nucleus with the pocket protein (Verona, R. et al1997; Iavarone, A et al 1999). This means, in the absence of pocket proteins they are cytoplasmic and do not have any transactivating property *in vivo*. When associated with pocket proteins they are nuclear but still can not activate any promoter due to the repression by pocket proteins and the chromatin remodeling factors recruited.

In contrast to activating E2Fs, repressive E2F–DP–pocket-protein complexes repressed E2F-responsive genes. It was seen that in G_0 and early G_1 phases of cell cycle, promoters were mostly occupied by E2F4, p107 and p130. Additionally, mutations of the E2F-binding sites increased the amount of transcripts from known E2F-responsive genes (Dalton, S et al 1992; Lam, E. W et al 1993; Hsiao, K. M et al 1994; Takahashi, Y et al 2000; Wells, J. et al 2000). In later stages the repressive E2F-pocket protein complexes were replaced by

activating ones. Consequently, there seems to be a two-level regulation of E2F-responsive genes by activating and repressive E2Fs; association/dissociation dependent regulation and transcriptional activation dependent regulation.

1.1.5.3 E2F6

E2F6 is the only member of this group. It has repressive effects as the repressive E2Fs. However there are other molecules regulating E2F6 function, instead of pocket proteins and recruited chromatin remodeling complexes. Binding of Ring1 and YY1 binding protein and Bmi-1 which are mammalian Polycomb (PcG) complex components, was thought to be the mediators of E2F6 functions. The structural difference of E2F6 prevented its regulation by pocket proteins, because it lacks all domains excluding DNA binding and dimerization domains found in other E2Fs. Lacking the transactivation region, E2F6 repressed E2F-responsive genes (Trimarchi, J. M. et al 1998; Cartwright, P et al 1998; Gaubatz, S et al 1998).

1.1.6 E2F1-RESPONSIVE GENES

There are a pack of genes having E2F binding sites (Table 1). As mentioned previously, repressive E2Fs mostly repress and activating E2Fs activate transcription of these genes when overexpressed. Being an intensively studied activating E2F, E2F1 was found to activate a variety of genes having different functions such as induction of proliferation, DNA synthesis and apoptosis. Such variety of genes suggests dual roles for E2F1 in regulation of cell cycle. It was shown before that overexpression of E2F1 in different systems could cause apoptosis or proliferation and transformation. *Jeffrey M. Trimarchi and Jacqueline A. Lees* proposed a threshold model for dual functions of activating E2Fs. The summary of this model is that there is a pool of active E2Fs in each cell. When this pool of active E2Fs reaches a first threshold level it activates proliferation. The second threshold level is later than the first one, and indicates problems in the cell. When the pool of active E2Fs reaches second threshold, they activate apoptotic genes. Seeming a good model, this scenario has not been

proven yet. It is also questionable what prevents activation of apoptotic genes before reaching the second threshold level.

It was shown that induction of apoptosis by E2F1 over-expression was mediated by $p14^{ARF}$, transcription of which was induced by E2F1. $p14^{ARF}$ protein caused stabilization and accumulation of active p53 by inhibiting the MDM2. Consequently p53 induced apoptosis. However apoptosis was induced by E2F1 overexpression in $p14^{ARF}$ and p53 deficient systems, suggesting other apoptotic mechanisms independent of p53. There are data suggesting another p53 family member as a candidate for induction of p53 independent apoptosis. P73 was shown to be directly transactivated by E2F1, and induced apoptosis in p53 deficient cell lines (Stiewe & Putzer 2000; Irwin et al 2000). Induction of apoptosis in T-cells by TCR activation was shown to be mediated by the p73 which was upregulated by E2F1 (Lissy et al 2000).

Table 1: The genes identified to have putative E2F1 binding sites on their promoters (*)

Gene name	Direct/ Indirect Induction	Function	Description	Reference
Thymidine Kinase	n.d	Replication		**
Thymidylate synthetase	n.d	Replication		**
ORC1	n.d	Replication		**
ORC6	n.d	Replication		**
cyclin A	n.d	Cell Cycle regulation		Ψ
CDC2	n.d	Cell Cycle regulation		Ψ
CDC25A	n.d	Cell Cycle regulation		Ψ
P107	n.d	Cell Cycle regulation	Retinoblastoma gene homolog	Ψ
Rb	n.d	Cell Cycle regulation	Retinoblastoma gene	Ψ
c-myc	n.d	Cell Cycle regulation		Ψ
N-myc	n.d	Cell Cycle regulation		Ψ
E2F1	n.d	Cell Cycle regulation	Activating E2F family member 1	Ψ
E2F2	n.d	Cell Cycle regulation	Activating E2F family member 2	
P14ARF	Direct	Apoptosis		¥
TP73	Direct	apoptosis, development	Tumor protein p73	§
B-Myb	n.d			
DHFR	n.d	cell cycle	Dihydro Folate Reductase	
DNA polymerase alpha	n.d	cell cycle		
Cdc6	n.d	Cell Cycle control	Limiting component of pre-replication complex	¶
CCND1	Direct	Cell Cycle control	cyclin D1	§
CCNE1	Direct	cell cycle control	cyclin E1	§
CCNE2	Direct	cell cycle control	cyclin E2	§
Map3K5	n.d	Others	Mitogen activated protein kinase kinase kinase 5	§
CD9	Indirect	Others	CD9 antigen (p24)	§
ENO2	n.d	Others	enolase2(neuronal)	§
IFNA2	n.d	Others	Interferon alpha2	§
KIAA0455	Indirect	Others	KIAA0455 gene product	§
KIAA0767	Direct	Others	KIAA0767 gene product	§
SERPINF2	n.d	Others	Serine (or cysteine) proteinase inhibitor, clade F (alpha-antiplasmin, pigment epithelium derived factor), member 2	§
UNG2	n.d	Others	Uracil-DNA glycolase 2	§
FGF-2	n.d	Cancer related	Fibroblast growth factor 2	§
FGFR3	n.d	Cancer related	Fibroblast growth factor receptor 3	§
MMP16	Indirect	Cancer related	matrix metalloproteinase 16	§

TP53BP2	n.d	Cancer related	tumor protein p53-binding protein	§
VEGF-B	Indirect	Cancer related	Vascular endothelial growth factor	§
BAD	n.d	Apoptosis	BCL2-antagonist of cell death	§
BAK1	n.d	Apoptosis	BCL2-antagonist /killer 1	§
BIF	n.d	Apoptosis	BH3 interacting domain death agonist	§
CFLAR (FLIP)	n.d	Apoptosis	CASP8 and FADD-like apoptosis regulator	§
MAP3K14	Direct	Apoptosis	Mitogen activated protein kinase kinase kinase 14	§
ARHGAP4	Indirect	Cell Cycle control/DNA replication/ centrosome duplication	Rho GTPase activating Protein	§
RAD52	Direct	DNA replication	RAD52 (S.cerevisiae) homolog	§
RFC3	Indirect	DNA replication	replication factor C (activator 1) 3	§
STK15	Indirect	Cell Cycle control/DNA replication/ centrosome duplication	serine threonine kinase 15	§
TNSF9	n.d	Cell Cycle control/DNA replication/ centrosome duplication	Tumor Necrosis factor superfamily, member 9	§
TRA1	Indirect	Cell Cycle control/DNA replication/ centrosome duplication	tumor rejection antigen (gp96) 1	§

*)Some of these genes were shown to be direct and indirect targets of E2F1. Some of them are not yet shown to be activated by E2F1 experimentally.

**) Helin et al 1998

Ψ) Slansky et al 1996

§) Stanelle et al. 2002

¥) DEGregori et al 1997; Bates et al 1998

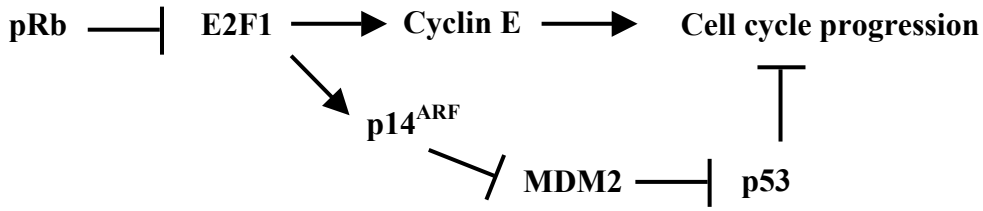
¶) Dyson et al 1998

1.2 p53 FAMILY OF PROTEINS

p53 is a tumor-suppressor protein, which was found to be inactivated in 50% of human cancers studied, as a result of mostly missense point mutations. There were also cases in which p53 was functionally inactivated by some viral oncogenes. It was activated by oncogenic activation of DNA damage. Post translational modification of p53 as a result of such stimuli stabilizes the protein and cause structural modifications on p53 , which allows it to oligomerize, bind to DNA and activate its target genes including p21(CIP) and Bax controlling cell cycle and apoptosis respectively (Giaccia and Kastan 1998). These modifications are phosphorylation and acetylation of the protein mainly at amino-terminal regions and carboxy-terminal regions respectively.

Activation of p53 is under a strict regulation due to its lethal effects on the cell. In normal cells its half-life is very short and its degradation is under the control of ubiquitin ligase MDM2, which targets p53 to ubiquitin dependent proteolysis. MDM is constitutively bound to p53 in its unphosphorylated state. Phosphorylation of N-terminal residues of p53 causes release of p53 from MDM2. Free p53 which is stabilized and activated transactivates its target genes, which induce either cell cycle arrest or differentiation or apoptosis. Being a critical protein for cell life p53 is an inducer of its own assassin MDM2. The levels of p53 in the cell is balanced by expression of MDM2 transactivated by p53 itself.

Being a very complex formation, life again put another guard over the MDM2. One of the most interesting loci identified is the multiple tumor suppressor locus (MTSL), encoding two different proteins, both of which are found to be anti-proliferative, and using alternative promoters. $p16^{INK4A}$ and $p14^{ARF}$ are these two protein products (Serrano M. 2000). $p14^{ARF}$ was identified as an inhibitor of MDM2, so over-expression of $p14^{ARF}$ resulted in p53 stabilization and activation of p53 target genes. As expected, $p14^{ARF}$ deficient mice developed tumors similar to p53 deficient mice did. There are several factors controlling $p14^{ARF}$ transactivation, one of which is the E2F1. This proposes a model for oncogenic activation of p53 as summarized below:



Sayan E. PhD Thesis, 2002

Figure 3: Dual roles of E2F1 in activation of cell progression and activation of p53

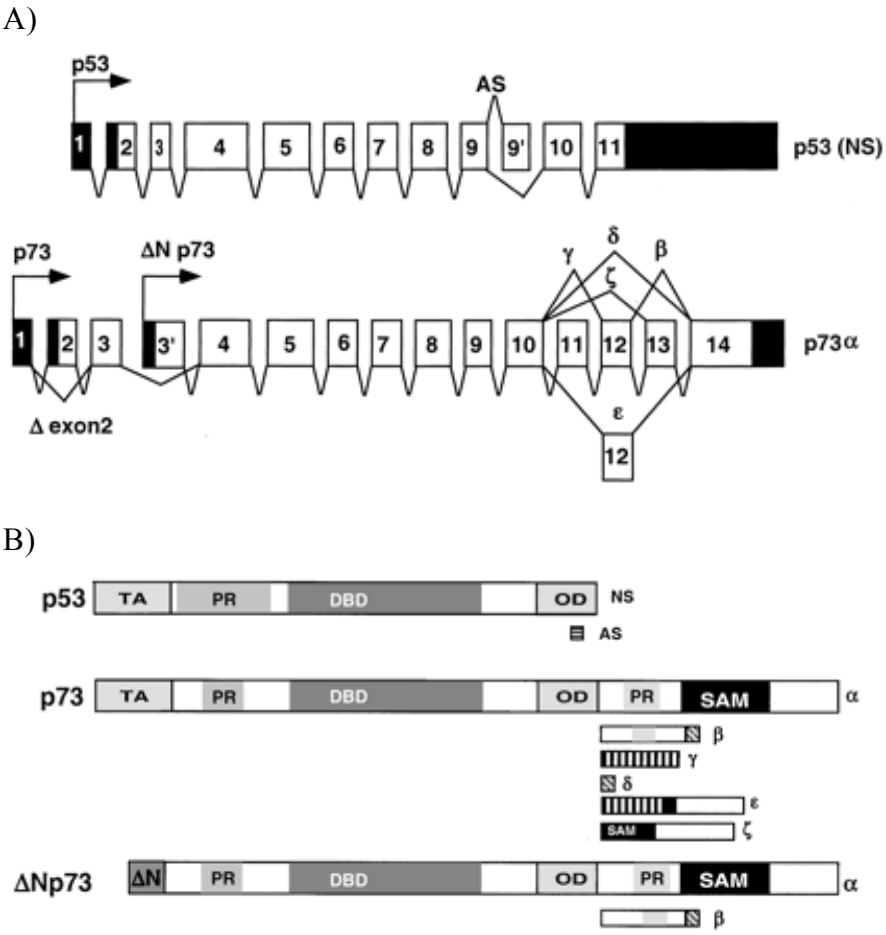
1.2.1 p73

Until 1997, it was thought that there was not any homolog of p53 gene. Kagdad and his colleagues identified the first sibling of p53 in a hybridization screen of COS cell line (Kaghad et. al 1997). Gene was localized to small arm of chromosome 1, deletion of which was common in different tumor types such as neuroblastoma (Kagdad et al 1997), for lung (Nomoto et al. 1998), for non-astrocytic brain tumors (Alonso et al. 2001), and for HCC (Mihara et al. 1999), suggesting a tumor suppressor role for the new p53 homolog, p73.

p73 has high homology with functional domains present in p53 protein (Kagdad et al 1997). There are 60%, 38%, and 29% homologies in DNA binding, oligomerization and transactivation domains respectively, between p53 and p73 proteins. The DNA binding domain of p73 is not a target of mutations in tumors as its homolog in p53 (Kagdad et al 1997). High homology between p73 and p53 suggests similar functions for two proteins. C-terminal extension of p73, which is not present in p53, contains two domains called SAM and PS domains. SAM domain (Sterile Alpha Motif) is a putative protein-protein interaction region, found in many signaling proteins involved in developmental processes. Presence of this domain suggests roles for p73 in development.

Figure 4: Homology of p53 and p73

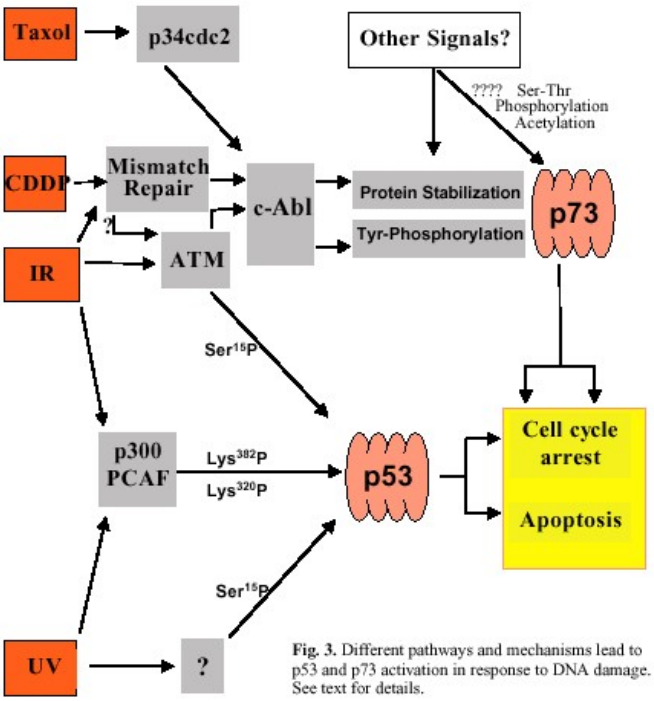
A distinct property of p73 gene from p53 gene is the presence of several N-terminal and C-terminal splice variants of p73 which are not present in p53. At least six types of C-terminal variants and at least three N-terminal variants are present (Figure 5) (Kaghad et al. 1997; Zaika et al. 1999). In normal tissues the p73α and P73β variants are abundant. It was shown that tumorigenesis led in these tissues accumulation of different transcripts. P73β was shown to have a potential of transactivation as much as p53 had. Following potential order was p73γ, p73α and p73ε. The other three forms (α, γ, ε) interestingly showed to endogenous p53 activity (Ueda et al. 1999). Different p73 forms could oligomerize together. This inter-association may result in different responses depending on the components of the oligomer. The N-terminal variants lacking transactivation domain and C-terminal variants having less transactivation ability may act as inhibiting factors for other variants in hetero-oligomers, as dominant negative forms. Actually such data were presented by Ueda et al showing that the p73 (p73γ, ε) isoforms decreased the transactivation potential of p53, p73α and p73β (Ueda et al). In another study it was shown that the expression of an N-terminal splice variant identified by Kaghad M. called Delta-exon2 was increased in breast cancer cell lines (Fillipovich et al. 2001) and in vulval cancers (O'Nions et al. 2001; Kaghad M et al 1997. DN-p53 transcript is initiated from an alternative in frame methionine in exon 3. It lacks the first 48 amino terminal amino acids, which are essential for transactivation. Conserving the DNA-binding and oligomerization domains, this form may oligomerize with other forms and bind to DNA.



Irwin M et al 2001

Figure 5: Organization of exons in different splice variants of p73 gene (A) and structural domains and comparison of different splice variants of p73 proteins (B)

Similar stimuli activating p53 could activate p73. Especially genotoxic stress, activated p73 in a c-Abl dependent manner, in which p73 was stabilized as a result of phosphorylation by c-Abl (Agami et al. 1999, Gong et al. 1999, Yuan et al. 1999). However there were differences in genotoxic agents activating p53 and p73. Cis-platin and ionizing radiation were the two agents activating p73 like p53. Activated p73 transactivated similar genes, those were activated by p53 such as Bax, p21, PIG series of genes, 14-3-3σ, a ribonucleotide reductase enzyme subunit and $p57^{KIP2}$, which are important actors in cell cycle regulation, DNA damage sensing, repair, and apoptosis (Stiewe and Putzer 2002).



Levrero et al 2000

Figure 6: Signals Activating p73 and p53

Posttranslational regulation of p73 was shown to be done also by MDM2 and its related protein MDMX (Ongkeko et al. 1999, Balint et al. 1999). The amino acid residues of p53 for MDM2 binding are conserved in p73, however binding of MDM2 and MDMX did not target p73 to degradation as MDM2 did p53. p73 was stabilized as a result of MDM2 and MDMX binding. This stabilization under the arms of MDMs did not increase but reduced the transactivation capacity of p73 (Zeng et al. 1999; Balint et al. 1999, Dobbelstein et al. 1999).

The C-terminal region which contain SAM domain and is not present in p53 suggests different functions for p73 in developmental processes. In a study with p73 knockout mice, it was shown that deficiency in p73 gene caused several developmental defects such as hydrocephalus, hippocampal dysgenesis and some secondary effects in pheromone sensory pathway, suggesting important roles for p73 in neurogenesis. Interestingly this study, in which the spontaneous tumor formation was not seen in p73 deficient mice, weakened the idea that p73 was a tumor-suppressor (Yang et al. 2000). Knock out studies provided discovery of an alternative promoter within the intron 3 of p73 gene encoding a p73 transcript

lacking the first three exons of p73 gene. This new form was named 'Dominant Negative' form due to the absence of transactivation domain found in full length p73. A similar form was identified for p63 the other member of the family. Further studies showed that the mRNA levels of DN-p73 were higher in developing and adult mouse tissues (Yang et al. 2000). Full length p73 was called Transactivating p73 (TA-p73) due to presence of p53-like transactivation domain at N-terminal region of the protein.

1.2.1.1 MUTATIONAL ANALYSIS OF p73

Being a homolog of p53, p73 was thought to be a tumor suppressor. However, screening of a legion of samples from different tumors revealed that just 0.5% of them had p73 mutations. This number is 100 times less than p53, which was mutated in 50% of tumors analyzed. P73 is thought to be deregulated at epigenetic levels in cancers. In many studies it was shown that p73 was expressed monoallelicaly or biallelically depending on the cell type, tissue, and person, but all these data were contradictory to each other (Stiewe and Putzer, 2002). There has not yet been found any correlation between the allelic expression of p73 and tumorigenesis.

In several cancers such as neuroblastomas, lung cancers, astrocytic and non-astrocytic brain tumors and hepatocellular carcinomas LOH incidence at 1p36 was reported to be quite high (**Table 2**).

A total of 11 polymorphisms were defined recurrently in different types of cancers (**Table 3**). Although 11 different polymorphisms of p73 were identified only two of them were shown to be associated with tumor progression. (Ryan et al.2001), It was proposed in this study that change in the stem like secondary structures in p73 mRNA as a result of different polymorphisms might affect translational efficiency of the p73 mRNA.

The number of mutations detected up to now is just 15. Being most of them point mutations causing amino acid substitutions, 3 deletions were also identified. Interestingly two of these deletions do not affect the reading frame. The hotspots (codons 175, 248, 249, etc) in p53 were interestingly not targeted by p73 mutations.

Table 2 : Summary of the studies defining the LOH at 1p36, mutation of p73 gene, polymorphisms of p73 gene and the expression of p73 RNA or protein (if otherwise is not indicated, it is RNA).

The bold lines are the references that we could obtain. The data for others are gathered from abstracts and the articles that cite them.

NT: Not Tested, T: tumor, N: Normal, +: positive, +/-: slightly positive, -: negative, prot: protein

Ref.	Samples	Number	LOH	Mutation	Polymorp.	Expression
Kaghad etal. 1997	**various**	**17**	**NT**	**1(neurolastoma)**	**2**	**NT**
Nomoto et al. 1998	lung ca.	62	42%(11/26)	none	6	NT
Takahashi et al. 1998	**prostatic ca.**	**106**	**6%**	**none**	**found**	**T>N (α>β)**
Mai et al. 1998 (a)	**lung ca.**	**21**	**NT**	**none**	**6**	**T>N**
Sunahara et al. 1998	**colorectal ca.**	**82**	**17%(8/46)**	**none**	**3**	**T>N**
Mai et al. (b) 1998	oligodendrioma	20	NT	none	found	
Nimura et al. 1998	**esophageal ca.**	**48**	**8%**	**none**	**found**	**T=+(α>β)**
Kovalev et al. 1998	neuroblastoma	42	NT	none	4	
Tsao et al. 1999	**melanoma**	**24**	**NT**	**none**	**9**	**NT**
Kroiss et al. 1998	melanoma	17	NT	none		
Ichimiya et al. 1999	**neuroblastoma**	**151**	**19%**	**2**	**4**	**T=+/-(α>β)**
Yokomizo et al. (a) 1999	**bladder ca.**	**30**	**NT**	**none**	**6**	**T>N (α, β)**
Han et al. 1999	**various**	**185**	**NT**	**1(breast)**	**4**	**NT**
Yokomizo et al. (b) 1999	prostate	31	NT	none		T=N
Yoshikawa et al. 1999	**various**	**54**	**NT**	**3(lung)**	**5**	

Herbst et al. 1999	**melanoma**	**56**	**6%(/17)**	**NT**	**NT**	
Shishikura et al. 1999	breast	87	13%	none		T=N
Chi et al. 1999	**bladder**	**45**	**NT**	**none**	**found**	**T>N(x1-x3)**
Zaika et al. 1999	**breast**	**8**	**NT**	**none**	**2**	**T>N(x2-x5)**
Stirewalt et al. 1999	leukemia	60		none	found	
Schittek et al. 1999	**melanoma**	**68**	**20%**	**none**	**NT**	**T>N**
Mihara et al. 1999	**HCC**	**48**	**20%**	**none**	**4**	**T=N(α>β)**
Corn et al. 1999	**leukemia/lymphoma**	**35**	**NT**	**none**	**4**	**T<N(x1-x3)**
Kawano et al. 1999	**leukemia/lymphoma**	**115**	**NT**	**none**	**2**	
Yokozaki et al. 1999	**gastric adenoca**	**95**	**38%**	**none**	**found**	**NT**
Liu et al. 2000	neuroblastoma	31	NT	none	found	
Schwartz et al. 1999	**breast**	**77**	**NT**	**none**		**NT**
Van Gele et al. 2000	**Merkel cell ca.**	**15**	**NT**	**1**	**4**	**NT**
Cai et al. 2000	**esophageal**	**15**	**64%**	**none**	**1**	**T>N**
Ng et al. 2000	**ovarian**	**70**	**50%(5/10)**	**none**	**NT**	**T>N(prot)**
Kang et al. 2000	**gastric adenoca.**	**80**	**-**	**none**	**NT**	**T>N**
Peng et al. 2000	HCC	22	18%	1(5bp del)		
Kong et al. 2000	neuroblastoma	50	38%	none	NT	
Tsujimoto et al. 2000	oligodendroglioma	10	NT	none	found	
Dominguez et al. 2000	breast	193	27%	NT	NT	
Ahomadegbe et al. 2000	**breast**	**59**	**32%**	**none**	**found**	**T>N**

Fukushima et al. 2001	HCC	36		none		
Shan et al. 2001	Parathyroid adenoma	32	37%	none		
El-Naggar et al. 2001	**oral/laryngeal ca.**	**67**	**30-40**	**2**	**1**	**N=T(prot)**
Nozaki et al. 2001	meningioma	27	NT	none		T>N
Alonso et al. 2001	**non-astrocytic**	**65**	**50%**	**1**	**3**	
Ichimiya et al. 2001	neuroblastoma	272	28/151	2 (1 germline)	ND	
Barrois et al. 2001	neuroblastoma	61	7/20	NT	NT	
Alonso et al. 2001	astrocytic	60	20%	none	5	
Dominguez et al. 2001	**breast**	**70**	**17%**	**NT**	**NT**	**T>N**
Lomas et al. 2001	**meningioma**	**30**	**NT**	**1**	**NT**	**NT**
F-Laurens et al. 2001	**HNSCC**	**17**	**NT**	**none**	**1**	**N=T**
Peters et al 2001	fam. prostate-brain	49	NT	none	found	
Momoi et al.2001	cholangiocarcinoma	23	high			
Ryan et al. 2001	oesoophageal	84	14/37			
Araki et al. 2002	squamus	41	73%	none		
Weber et al. 2002	HNSCC	68	ND	none		
Dong et al. 2002	**Oligodendroglioma**	**44**	**NT**	**1**	**5**	**T<N**

Table 3: The summary of all identified mutations and polymorphisms of the p73 gene with references.

5'UTR-ATG :	**A/G at nt 4 of ex 2**	**:** Kaghad et al. 1997, Nomoto et al. 1998, Mai et al. 1998 (a), Tsao et al. 1999, Yokomizo et al. (a) 1999
	T/C at nt 14 of ex 2	**:** Kaghad et al. 1997, Nomoto et al. 1998, Mai et al. 1998 (a), Tsao et al. 1999, Yokomizo et al. (a) 1999
Codons 101-200:	**S110L**	**:** Van Gele et al. 2000
	173(ACT/ACC)	**:** Mai et al. 1998 (a), Tsao et al. 1999, Yoshikawa et al. 1999, Cai et al. 2000, Lomas et al. 2001
	146(CCG/CCA)	**:** Alonso et al. 2001 (a), Lomas et al. 2001
Codons 201-300:	**R269Q**	**:** Han et al. 1999
	245(GTG/GTA)	**:** Yoshikawa et al. 1999, Zaika et al. 1999, Corn et al. 1999
	G264W	**:** Yoshikawa et al. 1999
	Q291K	**:** Alonso et al. 2001 (a)
	204(AAC/AAT)	**:** Alonso et al. 2001 (a), Lomas et al. 2001, F-Laurens et al. 2001
	N204S	**:** Lomas et al. 2001

Codons 301-400 : **336(GCC/GCT)** **:** Nomoto et al. 1998, Mai et al. 1998 (a), Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichimiya et al. 1999, Yokomizo et al. (a) 1999, Yoshikawa et al. 1999, Kawano et al. 1999, Van Gele et al. 2000, Lomas et al. 2001

349(CAT/CAC) **:** Nomoto et al. 1998, Mai et al. 1998 (a), Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichimiyaa et al. 1999, Yokomizo et al. (a) 1999, Yoshikawa et al. 1999, Corn et al. 1999, Kawano et al. 1999, Van Gele et al. 2000, El-Naggar et al. 2001, Lomas et al. 2001

Codons 4010500: **P405R** **:** Ichimiya et al. 1999, Zaika et al. 1999

P425L **:** Ichimiya et al. 1999

Del 2 and 4 bp in coding exon 10 affecting codons 417-420 **:** Yoshikawa et al. 1999

S469R **:** El-Naggar et al. 2001

A472T **:** Kaghad et al. 1997

S477W **:** El-Naggar et al. 2001

Codons 501-636 : **557(GCG/GCA)** **:** Nomoto et al. 1998, Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichimiya et al. 1999, Yoshikawa et al. 1999, Corn et al. 1999, Van Gele et al. 2000

563(TCT/TCC) **:** Yoshikawa et al. 1999

610(GCG/GCA) **:** Nomoto et al. 1998, Mai et al. 1998 (a), Nimura et al. 1998, Tsao et al. 1999, Mihara et al. 1999, Ichimiya et al. 1999, Yokomizo et al. (a) 1999, Yoshikawa et al. 1999, Corn et al. 1999, Van Gele et al. 2000

Del 12 bp at coding exon 13, so deletion of codons 604-606 **:** Yoshikawa et al. 1999

1.2.1.2 Transactivating p73 (TA-p73)

The p73 locus seems to encode mainly two classes of proteins, with regard to presence of transactivation domain at N-terminal or not. The first class includes the TA-p53 and its C-terminal variants. The second group includes the DN-p73, Δexon2, Δexon2-3 spliced forms and their C-terminal variants.

Endogenous expression of TA-p73 and its C-terminal variants showed p53 like properties. Oligomerization, binding to p53 response elements, and transactivation of several genes having role in cell cycle regulation and apoptosis (such as p21, 14-3-3-σ, PIG series (PIG3, PIG6, PIG7 and PIG11), MDM2, a ribonucleotide reductase p53R2) are the p53-like biological activities of TA-p73 (Zhu et al 1998; Nakano et al. 2000).

The probable pro-apoptotic and cell cycle regulatory role of TA-p73 is cell type dependent. TA-p73, like some other cell cycle regulatory proteins also regulates the differentiation state of different cell types.

Activation of TA-p73 may cause different responses depending on the cell type. Retinoic acid treatment with over-expression of TA-p73 of neuroblastoma cell line, induced morphological and biochemical markers of neuronal differentiation (De-Laurenzi et al. 2000) whereas neither p53 nor DN-forms of p73 could cause any change in differentiation status of cells. In EJ bladder carcinoma cells TA-p73α and TA-p73β caused irreversible growth arrest together with the markers of replicative senescence when over-expressed. The effects of TA-p73α and TA-p73β were quite similar of neuroblastoma over-expression in bladder cells (Fang et al. 1999).

1.2.1.3 Dominant negative p73 (DN-p73)

Transactivation domain of TA-p73, which has role in induction of cell cycle arrest and apoptosis is absent in DN-p73. The proposed role for DN-p73 is being the antagonist of TA-p73, its C-terminal isoforms and maybe p53. TP73 has an interesting gene architecture, in which two groups of proteins are encoded,

one seem to be antagonist (one is probably oncogenic and the other is tumor suppressive) of the other with the regulation under distinct promoters.

There is not quite much information about the function of DN-p73. Withdrawal of Nerve Growth Factor (NGF) induced a p53 dependent apoptosis in sympathetic neurons. In sympathetic neurons, when NGF (Nerve growth factor) was withdrawn, apoptosis was induced in a p53 dependent manner. The protein levels of DN-p73 were decreased suggesting a balance between p53 and DN-p73 on apoptosis. This idea was strengthened by the rescue of these cells from apoptosis with adenoviral transfection of DN-p73 after NGF withdrawal (Pozniak et al. 2000). Similarly infection of neuronal cells with both p53 and DN-p73 together did not lead these cells to go apoptosis. Pull down assays showed that these two proteins form complexes in vivo, supporting their antagonist activity (Pozniak et al. 2000).

1.2.2 INHIBITION OF p73 BY p53 MUTANTS

There are several oncogenic stimuli leading to upregulation of p53 and TA-p73 such as E2F1 upregulation (as a consequence of Rb Pathway dysregulations including, Rb mutation, pRb degradation, p16 gene mutations and promoter methylations). Upregulation of TA-p73 in response to E2F1 or stabilization of TA-p73 by c-Abl and over-expression induced apoptosis in cells (Stiewe et al. 2000, Jost et al. 1997, Gong et al. 1999). As expected it was seen that as a consequence of tumorigenesis the levels of TA-p73 was increased in many tumors except leukemias and lymphomas. Although it is not very favorable, it is very common that TA-p73 expression is prolonged in cancer cells. It was found that some mutant forms of p53 could inhibit the probable apoptotic effect of TA-p73 by direct protein-protein interaction. The cancer cells might generate transactivation defective oligomers of TA-p73 and mutant p53, which can not activate apoptotic genes. Direct interaction of TA-p73α and two mutant forms of p53 (R175H and R248W mutants) was shown in a co-transfection experiment with co-immunoprecipitation (Di Como et al. 1999). In the same study activity of TA-p73 was shown to be decreased with p53 mutants. The interaction between the mutant p53 and TA-p73 was not mediated by oligomerization domains but

with a peptide motif in DNA binding domain, with the oligomerization domain (Davison et al. 1999; Strano et al. 2000, Gaiddon et al. 2001).

1.2.3 ONCOGENIC ACTIVATION OF p73

The upstream pathways inducing p73 activation may give clues about the role of p73. One of the striking discoveries about regulation of p73 was the induction of p73 gene in transcriptional level by over-expression of some oncogenes. Two Nature and a Nature genetics papers, showed that the induction of p73 in response to E2F1 activation caused apoptosis. Lissy *et al* showed that the TCR-AICD (T-cell receptor activation induced cell death) which had been shown to be independent of p53, was mediated by the activation of p73, in response to activation of E2F1 transcription factor (Lissy NA et al 2000). The second paper in the same issue of Nature by Irwin *et al* was about the transcriptional activation of p73 in response to over-expression of E2F1. E2F1 was shown to increase both mRNA and protein levels of p73 with northern blot and western blot analysis respectively. P73 promoter was shown to be E2F1 responsive with reporter assays. Interestingly it was shown that different members of E2F1 family had different dose dependent affinities on p73 promoter. E2F1 and E2F4 were shown to be the most and less potent activators of the p73 promoter respectively. The physiological regulation of E2F1 during cell cycle also correlated with the amount of p73 transcripts at different periods of cell cycle in a starvation-refeeding experiment. A dominant negative form of p73 p73DD, was shown to decrease apoptosis in Saos-2 cells (p53 deficient) transfected with E2F1, suggesting p73 as the mediator in the E2F1 induced apoptosis. The last experiment with MEFs showed that, the p53-/- or p73 -/- MEFs had a significant decrease in percentage of apoptosis (from 80% to 15%) in response to E2F1 transfection. Thorsten Stiewe and Brigitte M. Putzer used another approach to show the effect of E2F1 on p73 transcription and induction of apoptosis. The increase in the RNA and protein levels were shown by semi-quantitative RT-PCR and western blots respectively. A tumor derived p53 mutant, which directly inhibits p73 and interferes with its transactivation function, was shown to reduce E2F1 mediated apoptosis. "These results suggest

that deregulated E2F1 activity might constitute a p53-independent, anti-tumorigenic safeguard mechanism" (Stiewe T, & Putzer B 2000)

Additional oncogenes such as c-myc and E1A were shown to induce transcription of p73. It was shown that in p53 deficient tumor cells, the endogenous levels of p73α and p73β were shown to be induced in response to E2F1, c-myc and E1A over-expression. Using p73 responsive reporter activity and known endogenous p73 target genes, increase in the levels of p73 transcription activity was shown with again over-expression of oncogenes. As E2F1, c-myc and E1A were shown to induce apoptosis. Apoptotic effect of these oncogenes was demonstrated to be mediated by p73, using dominant negative p73 protein, which decreased apoptosis level in E1A or c-myc transfected Saos-2 cells (Zaika A. et al 2001)

1.3 AIM OF THE STUDY AND STRATEGY

Having putative opposing roles, TA- and DN- forms of p73 encoded in the same gene with distinct promoters makes p73 gene an interesting target for tumorigenesis studies. TA-p73 which was expected to induce apoptosis was somehow ineffective in cancer cells, although its expression was elevated in response to different stimuli including oncogenic activation. In a previous study Sayan *et al* showed that in normal liver tissue the dominant negative form was expressed but TA-p73 was not. However in 14/15 of cell lines and 3/7 tumor samples of HCC, an acquired expression of TA-p73 was detected with semi-quantitative RT-PCR. It was thought that this acquired expression might be a consequence of E2F1 activation due to pRb pathway dysregulations. It was shown with p14 and p16 semi-quantitative PCRs and western blotting for pRb that in most of the samples pRb pathway seemed to be dysregulated, correlating with the TA-p73 activation. On the other hand p53 of most cells were found to have missense mutations or loss of expression. The mutant forms of p53 and DN-p73 expressed in these cells could be factors neutralizing apoptotic effects of acquired TA-p73 expression.

It was also shown that E2F1 induced p53-independent apoptosis using TA-p73 as a mediator in different cell types. However it is still not clear why and how acquired expression of TA-p73 is favored without induction of apoptosis in cancer cells. In the studies linking E2F1 and p73 to apoptosis it was shown that only TA-forms of the p73 were activated. However it has not yet been shown whether DN-form or other transactivation domain lacking forms (p73-Δexon2 and p73-Δexon2-3) are the targets of E2F1. Such a dual role could be an advantageous way for cancer cells to overcome apoptotic effects of active TA-p73. E2F1 dependent activation of different splice variants of p73 may also be cell type specific and in different cell types E2F1 over-expression may give different responses. In order to show which forms of p73 are activated in response to E2F1 over-expression we selected different cell lines having different background of p53, pRb, p14, p16, and p73 status. With semi-quantitative RT-PCR, changes in the expression levels of different forms were detected. E2F4 transfected and untransfected cells were used as negative controls. Later on, time-

course activations of TA-p73 and DN-p73 forms were demonstrated using semi-quantitative RT-PCR. This time just Cama-1 cell line was used. Cama-1 was a good candidate because neither DN-p73 nor TA-p73 forms seemed to be expressed in untransfected cells and a time course activation of p73 could be demonstrated quite well in such a system. It may be important in what sequence different forms are activated in determining the cell fate during tumorigenesis.

CHAPTER 2

MATERIAL

2.1 COMMERCIAL KITS

QIAGEN Maxi-Prep Kit

MN's Nucleospin Mini-Prep Kit

MN's Nucleobond RNA isolation Kit

MBI's RevertAid cDNA Synthesis Kit

MBI's Recombinant Taq Polymerase PCR kit

ECL+ Immunodetection Substrate Kit

Promega's pGEM-T TA-cloning Kit

2.2 BACTERIAL STRAINS

Strain	Genotype	Usage	Reference
DH5α	*supE*44 Δ*lac*U169 (ϕ80*lacZΔ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Host for plasmid DNA	Hanahan (1983)

2.3 PLASMIDS AND CONSTRUCTS

pCDNA3- TAp73	TA-p73 is cloned into pCDNA3 vector from cDNA (Kindly provided by T. Soussi, France).
pRC/CMV- E2F1	E2F1 is cloned into pRC/CMV vector from cDNA (Kindly provided by R. Bernards, Netherlands)
pRC/CMV- E2F4	E2F4 is cloned into pRC/CMV vector from cDNA (Kindly provided by R. Bernards, Netherlands)
pRC/CMV-p53	Wild Type p53 is cloned into pRC/CMV vector from cDNA (Kindly provided by T. Frebourg, France)
pEGFP-N2	Encodes GFP protein (Clontech)
pGEM-T	TA-cloning vector for sequence analysis (Promega)

(For extra information look at the Appendix)

2.4 SOLUTIONS AND BUFFERS

LB medium

Tryptone	10 gr
Yeast Extract	5 gr
NaCl	10 gr
Agar	20 gr (For Plates only)

Complete volume to 1 L with dd H2O, and autoclaved

SOB Medium

Per Liter;

2% Tryptone
0.5% Yeast extract
10mM NaCl
2.5mM KCl
} Autoclaved

Then 20 mM $MgSO_4$ and 10mmM $MgCl_2$ are added.

SOC Medium

SOB+20mM Glucose (from filter sterilized 1M stock solution in ddH_2O)

Ampicilline

Working Solution: 100µg/ml

Stock solution: 100 mg/ml

Kanamycine

Working solution: 25µg/ml

Stock solution : 25 mg/ml

EDTA 0.5 M (pH:8.0)

For 1L

186.1 g EDTA is dissolved in 800 ml ddH_2O.

pH is adjusted to 8.0 and volume is completed to 1L with ddH_2O.

Solution I

50 mM glucose

25 mM Tris.Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution II

0.2M NaOH (freshly diluted from 1 M stock)

1% SDS

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H2O	28.5 ml

TE buffer pH 7.4

10mM Tris.Cl (pH 7.4)

1mM EDTA (pH 8.0)

Buffer TB, for supercompetent cell preparation

10 mM PIPES

55mM $MnCl_2$

15 mM $CaCl_2$

250 mM KCl

pH 6.7

Filter Sterilized

Growth Medium for Mammalian Cells

500 ml DMEM

50 ml FBS

5 ml 100X Non-Essential amino acid mix

5 ml 100X Penicilline/Strpetomycine ((10000 units/ 10000µg)/ ml)

All are sterile.

10 X PBS (Phosphate Buffered Saline)

80 g NaCl

2 g KCl

11.5 g Na_2HPO_4

2.4 g KH_2PO_4

Volume is adjusted to 1 Lt with ddH_2O

1X PBS Buffer

10X PBS is diluted in 9 volumes of ddH_2O and pH is adjusted to 7.3-7.6.

CaCl2 2.5 M (stored at -20°C)

CaCl2 50mM (stored at +4°C)

2 X BES Buffer

50 m BES

280mM NaCl

1.5 mM Na_2HPO_4

pH is adjusted to 6.95, Filter sterilized, Stored at -20°C

5X RNA Running Buffer (for 500ml)

10 ml of 2M Sodium Acetate

10.3 gr MOPS

390 ml DEPC treated ddH_2O are mixed and pH is adjusted to 7.0 with Sodium Hydroxide

5 ml, 0.5 M, pH. 8.0 EDTA is added.

Volume is completed to 500 ml with DEPC treated ddH_2O.

4***X RNA loading Buffer***

50% formamide

20% Formaldehyde

15% 5X running buffer

15% glycerol-dye

in DEPC treated ddH_2O. Stored at -20°C.

DEPC H2O

1 ml DEPC in 1 L ddH_2O

Stirred for 12 hours and autoclaved.

50X TAE Buffer

2M Tris base

57.1 ml Glacial Acetic acid

50 mM EDTA

1X DNA loading buffer

0.25% bromophenol blue

0.25% xylene cyanole

50% glycerol

1mM EDTA

Ethidium bromide solution

10 mg/ml in water (stock solution)

30 ng/ml (working solution)

2.5 PRIMERS

Annealing temperature

P73 C-terminal primers

P73-LF

(5'-GCCGGGATCCATATGGTGCCGCAGCCACTGGTGGAC-3') 64°C

TT2-P73END

(5'-CTCTCGAGAGTGGAT CTCGGCCTCCGTGAAC-3') 64°C

TA-p73 primers

1st set of primers (Fillipovich et al 2000)

Fillip-F

(5'-GGACGGACGCCGATGCC-3') 64°C

Fillip-R

(5'-GGTCCATGGTGCTGCTCAGC-3') 64°C

2nd set of primers

p73-VNF (Forward)

(5'-CCAGGCCAGCCGGGACGGA-3') 64°C

p73-VNR (Reverse)

(5'-CTTGGCGATCTGGCAGTAGA-3') 64°C

(Common reverse for both TA and DN Forms)

p73 DN form Primers

p73-DNF (New Forward) :

(5'-GCTGTACGTCGGTGACCCC-3') 62°C

p73-VNR (Reverse) :

(5'-CTTGGCGATCTGGCAGTAGA-3') 62°C

GAPDH Primers (sayan et al 2001)

GAPD-F

(5'- GGCTGAGAACGGGAAGCTTGTCAT-3') 62°C

GAPD-R

(5'- CAGCCTTCTCCATGGTGGTGAAGA-3') 62°C

P14 Primers (Sayan et al 2001)

$p14^{ARF}$-F

(5'- TCACCTCTGGTGCCAAAGGG -3') 62°C

C-R

5'- GGCAGTTGTGGCCCTGTAGG -3') 63°C

2.5.1 Alignment of the p73 primers on cDNA sequence of p73

```
   1 aggggacgca gcgaaaccgg ggcccgcgcc aggccagccg ggacggacgc cgatgccggg
                                      p73-VNF    Fillip-F primer

  61 ggctgcgacg gctgcagagc gagctgccct cggaggccgg cgtggggaag atggcccagt
 121 ccaccgccac ctcccctgat gggggcacca cgtttgagca cctctggagc tctctggaac
 181 cagacagcac ctacttcgac cttccccagt caagccgggg gaataatgag gtggtgggcg
 241 gaacggattc cagcatggac gtcttccacc tggagggcat gactacatct gtcatggccc

 (For only DN-p73)      accatgctg tacgtcggtg accccgcacg gcacctcgcc acg
                            DN-p73 Forward

 301 agttcaatct gctgagcagc accatggacc agatgagcag ccgcgcggcc tcggccagcc
                  Fillip-R primer

 361 cctacacccc agagcacgcc gccagcgtgc ccacccactc gccctacgca caacccagct
 421 ccaccttcga caccatgtcg ccggcgcctg tcatcccctc caacaccgac taccccggac


 481 cccaccactt tgaggtcact ttccagcagt ccagcacggc caagtcagcc acctggacgt
 541 actccccgct cttgaagaaa ctctactgcc agatcgccaa gacatgcccc atccagatca
                               p73-VNR (common)

 601 aggtgtccac cccgccaccc ccaggcactg ccatccgggc catgcctgtt tacaagaaag
 661 cggagcacgt gaccgacgtc gtgaaacgct gccccaacca cgagctcggg agggacttca
 721 acgaaggaca gtctgctcca gccagccacc tcatccgcgt ggaaggcaat aatctctcgc
 781 agtatgtgga tgaccctgtc accggcaggc agagcgtcgt ggtgccctat gagccaccac
 841 aggtggggac ggaattcacc accatcctgt acaacttcat gtgtaacagc agctgtgtag
 901 ggggcatgaa ccggcggccc atcctcatca tcatcaccct ggagatgcgg gatgggcagg
 961 tgctgggccg ccggtccttt gagggccgca tctgcgcctg tcctggccgc gaccgaaaag
1021 ctgatgagga ccactaccgg gagcagcagg ccctgaacga gagctccgcc aagaacgggg
1081 ccgccagcaa gcgtgccttc aagcagagcc cccctgccgt ccccgccctt ggtgccggtg
1141 tgaagaagcg gcggcatgga gacgaggaca cgtactacct tcaggtgcga ggccgggaga
1201 actttgagat cctgatgaag ctgaaagaga gcctggagct gatggagttg gtgccgcagc
1261 cactggtgga ctcctatcgg cagcagcagc agctcctaca gaggccgagt cacctacagc
     C-terminal Forward

1321 ccccgtccta cgggccggtc ctctcgccca tgaacaaggt gcacgggggc atgaacaagc
1381 tgccctccgt caaccagctg gtgggccagc ctcccccgca cagttcggca gctacaccca
1441 acctggggcc cgtgggcccc gggatgctca acaaccatgg ccacgcagtg ccagccaacg
1501 gcgagatgag cagcagccac agcgcccagt ccatggtctc ggggtcccac tgcactccgc
1561 caccccccta ccacgccgac cccagcctcg tcagtttttt aacaggattg gggtgtccaa
1621 actgcatcga gtatttcacc tcccaagggt tacagagcat ttaccacctg cagaacctga
1681 ccattgagga cctgggggcc ctgaagatcc ccgagcagta ccgcatgacc atctggcggg
1741 gcctgcagga cctgaagcag ggccacgact acagcaccgc gcagcagctg ctccgctcta
1801 gcaacgcggc caccatctcc atcggcggct caggggaact gcagcgccag cgggtcatgg
1861 aggccgtgca cttccgcgtg cgccacacca tcaccatccc caaccgcggc ggcccaggcg
1921 gcggccctga cgagtgggcg gacttcggct tcgacctgcc cgactgcaag gcccgcaagc
1981 agcccatcaa ggaggagttc acggaggccg agatccactg agggcctcgc ctggctgcag
                                  TT2-C-terminal Reverse

2041 cctgcgccac cgcccagaga cccaagctgc ctcccctctc cttcctgtgt gtccaaaact
2101 gcctcaggag gcaggacctt cgggctgtgc ccggggaaag gcaaggtccg gcccatcccc
2161 aggcacctca caggccccag gaaaggccca gccaccgaag ccgcctgtgg acagcctgag
2221 tcacctgcag aacc
```

2.5.2 Expeceted band lengths of differnt variants in PCR Reactions:

N-Terminal Splice variants

TA-p73 form:

With first set of TA-p73 primers : 290 bps
With second set of TA-p73 primers : 553 bps

Δexon2 spliced form:

With first set of TA-p73 primers : 171 bps
With second set of TA-p73 primers : 433 bps

Δexon2-3 spliced form:

With second set of TA-p73 primers : undetectable
With second set of TA-p73 primers : 187 bps

DN-p73 Spilce-Variant : 328 bps

C-Terminal Variants:

p73α (14 exons) : 766 bps

p73β (Δ exon 13) : 660 bps

p73γ (Δ exon 11) : 617 bps

p73ε (Δ exons 11, 13) : 510 bps

p73ϕ (Δ exons 11, 12) : 478 bps

p73δ (Δ exons 11, 12, 13 : 382 bps

2.6 CELL LINES

Cell Line	Origin	Status of Endogenous p53	pRb	p16	p14	TA-p73	DN-p73	References
CAMA-1	Breast CA	MT	+	-ve	+	-ve	-ve	(i)
Saos-2	Osteosarcoma	-ve	-ve	+	?	?	?	(ii)
Hep3B	HCC	-ve	-ve	+	+	+	+	(iii)
HepG2	HCC	W T	+	+	+	+	+	(iv)
SK-Hep1	HCC	WT	+	-ve	-ve	+	+	(v)
SNU398	HCC	-ve	+	weak	+	+	+	(vi)

(i) Sayan E unpublished data; Ji et al, 1994.
(ii) Chandar et al 1992, Shew et al 1990; Spillare et al 1996.
(iii) Sayan et al, 2001; Puisieux et al, 1993.
(iv) Puisieux et al, 1993; Sayan et al, 2001; Hsu et al, 1993.
(v) Hsu et al, 1993; Sayan et al, 2001.
(vi) Sayan et al, 2001.

CHAPTER 3

METHODS

3.1 PREPARATION OF SUPER-COMPETENT CELLS

DH-5α *E.coli* strain was used to prepare super-competent cells. Glycerol stock of DH5-α was grown overnight and diluted in 1 liter of SOB medium with a starting absorbance of 0.2 at 600 nm. Then super-competent cells were prepared as described in Inoue H *et al* 1990.

3.2 TRANSFORMATION

In super-competent cells 0.5 µg plasmid DNA was put and mixed with tapping with fingers. After 30 minutes incubation on ice the cells were shocked in 42°C water bath for 90 seconds. Shocked cells were kept in ice for 2 minutes and 800 µl SOC medium was added on the cells. Following 1 hour incubation at 37°C the cells were spread on antibiotic containing LB plates. Plates were incubated at 37°C for 16 hours and stored at +4°C for future use.

3.3 SMALL SCALE PLASMID ISOLATION (MINI-PREP)

Candidate colonies were inoculated in 5ml of LB medium with addition of appropriate antibiotic. After incubation at 37°C overnight with shaking the 1.5 ml of cultures was precipitated using micro-centrifuge. The cell pellet was resuspended in 100µl Solution 1 (pre-lysis solution) with RNAase I. Then 200µl Solution II (lysis buffer) was added and the tube was mixed gently by inverting several times. Solution III (neutralization buffer) was added and the tube was mixed gently by inverting several times. The mixture was kept on ice for 10 minutes and spinned at highest speed at 4°C for 10 minutes in micro-centrifuge. The supernatant was transferred into new 1.5 ml eppendorf tube and 2 volumes of cold absolute ethanol were added. Plasmid DNA was precipitated by centrifuging at 4° with highest speed for 15 minutes in micro-centrifuge. Then supernatant was discarded and pellet was washed once with cold 70% ethanol. The tube was left on benchtop for drying. Dried pellet was dissolved in 50 µl of sterile ddH_2O and kept at -20°C.

3.4 LARGE SCALE PLASMID ISLOATION (MAXI-PREP)

Bacterial cells were grown overnight at 37°C shaking incubator in 250 ml LB with appropriate antibiotic. Grown cells were centrifuged and from the cell pellet plasmid DNA was isolated using the QIAGEN maxi-prep kit as described in the manual of the kit. Each batch of isolation yielded a DNA amount of nearly 500-1000 μg.

3.5 SPECTROPHOTOMETRIC QUANTIFICATION OF DNA

Concentration and purity of the DNA solutions were determined using spectrophotometric measurement of the DNA samples. In new eppendorf tubes the DNA samples were diluted in 100 Volumes of ddH_2O and measurements were done using Beckman Instruments Du series 600 Spectrophotometer software programs (ds DNA in Oligo DNA short methods) on the Beckman Spectrophotometer Du 640 (Beckman Instruments Inc. CA. USA) at A_{260} and A_{280}.

3.6 STORAGE OF BACTERIAL CELLS

Cells were grown in LB medium containing appropriate antibiotic overnight. In sterile 1.5 ml eppendorf tubes equal volumes of bacterial culture and sterile 60% glycerol were mixed well using vortex. The mixture was stored at –80°C.

3.7 CELL CULTURE

Mammalian cells were used as sources for RNA for RT-PCR analysis. Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% Fetal Bovine Serum, 1mM L-Glutamine, non-essential amino acids and penniciline/streptomycine (100units/ 100ug/ml) mix. Cells were incubated at 37°C in an incubator with an atmosphere of 5% CO_2 in air.

Passage of the cells into new plates was done before reaching full confluence. Old medium was aspirated and cells were washed once or more with sterile 1X PBS. Trypsin was added on cell monolayer and incubated at 37°C for 20 seconds to 5 minutes depending on the cell type. Fresh medium was added on the cells and cells were dispersed well by pipetting gently. Dispersed cells were transferred into new dishes with new dilutions.

3.8 CALCIUM PHOSPHATE TRANSFECTION

In order to insert foreign DNA transiently into mammalian cells there are several methods like electroporation, lipofection and calcium phosphate transfection. For transient transfection the easiest way is the Calcium Phosphate method if optimized well. The cells were subcultured into 100mm dishes 24 hours before transfection aiming a confluency of 40% at transfection time. For this purpose 250.000 to 350.000 cells were cultured in each 100mm plate depending on the growth rate of the cell lines used. Transfections were done using 20µg subject plasmid and 10µg pEGFP-N2 plasmid. The plasmid DNA was dissolved in ddH_20 and then 50µl 2.5M $CaCl_2$ was added into the DNA solution drop by drop while vortexing the solution. Calcium chloride-DNA mixture was kept at room temperature for 30 minutes. During this time the mediums of the cells were changed. At the end of 30 minutes, 500 µl of 2X BES solution was added into the DNA-Calcium Chloride solution drop by drop while vortexing the solution. The mixture and the cells were kept at room temperature and 37°C respectively for at least 40 minutes. Then the DNA-Calcium Chloride-BES solution was spread on cells drop by drop. Cells were incubated at 37°C for 12-16 hours depending on the cell. The medium was changed. This time the cells were washed well before adding new medium in order to get rid of all extra calcium chloride-DNA complexes.

3.9 RNA ISOLATION

24 hour post-transfection the cells were washed with ice-cold PBS once. 350µl RA1 buffer of MN's RNA isolation kit was added on cells and cells were

scraped at 4°C. Lysates were put in RNAase-free eppendorf tubes and frozen immediately in liquid nitrogen and kept at -80°C for future use. For isolation of RNA from these lysates the procedure described in the manual of the kit was followed. Only modification done was the usage of 250µl absolute ethanol instead of 350 µl 70% ethanol added into the cell lysate. RNA samples were frozen immediately in liquid nitrogen for future use. Before freezing, samples for spectrophotometric and electrophoretic analysis were taken in separate tubes.

3.10 SPECTROPHOTOMETRIC QUANTIFICATION OF RNA

Concentration and purity of the RNA solutions were determined using spectrophotometric measurement of the RNA samples. In new eppendorf tubes the RNA samples were diluted in 100 Volumes of ddH_2O (RNAse free) and measurements were done using Beckman Instruments Du series 600 Spectrophotometer software programs (RNA measurement short methods) on the Beckman Spectrophotometer Du 640 (Beckman Instruments Inc. CA. USA) at A_{260} and A_{280}.

3.11 AGAROSE GEL ELECTROPHORESIS OF RNA SAMPLES

In 45 ml of 1X RNA running buffer 0.5 gram of agarose was dissolved. After boiling it in microwave oven, 10ml of formaldehyde and Et-Br were added and gel mixture was poured on the tray under hood in order to avoid formaldehyde inhalation.

3.12 cDNA SYNTHESIS

For each synthesis reaction 3µg RNA was used. cDNA synthesis was carried on as described in the MBI's RevertAid first strand cDNA synthesis kit manual. Synthesis products were diluted in 2 volumes of sterile ddH_2O and stored at –20˚C.

3.13 POLYMERASE CHAIN REACTION

Quantitative PCR reactions were used to compare the number of copies of a DNA fragment in a mixture. Before testing the samples the reaction should be optimized for each fragment will be tested in samples. Optimizations for GAPDH, p14, TA-p73, DN-p73, and C-terminal region of p73 PCRs were previously done by Emre Sayan. Each reaction was done in 50µl total reaction volume containing 5µl 10X PCR reaction buffer (NH4), 5 µl dNTP mix (2mM stock), 3µl $MgCl_2$ (2.5 mM stock), 1ul forward primer (25 picomolar), 1 µl reverse primer (25 picomolar), 0.3 µl Taq Polymerase (1 units/µl), 1.5 ml DMSO, and template DNA (amount depend on the fragment tested). cDNAs prepared were used as template. The volume was completed to 50µl with ddH_2O

The conditions for GAPDH PCR:

94˚C	5 minutes	
94˚C	30 seconds	24 cycles
62˚C	30 seconds	
72˚C	30 seconds	
72˚C	5 minutes	

Conditions for TA-p73 and C-terminal region PCRs:

94˚C	5 minutes	
94˚C	30 seconds	35 cycles
64˚C	30 seconds	
72˚C	30 seconds	
72˚C	5 minutes	

The conditions for p14ARF PCR:

94˚C	5 minutes	
94˚C	30 seconds	30 cycles
61˚C	30 seconds	
72˚C	30 seconds	
72˚C	5 minutes	

The conditions for DN-p73 PCR:

94˚C	5 minutes	
94˚C	30 seconds	35 cycles
62˚C	30 seconds	
72˚C	30 seconds	
72˚C	5 minutes	

AGAROSE GEL ELECTROPHORESIS

1% agarose was prepared by dissolving enough agarose in 1X TAE buffer and boiling in microwave oven. After adding Et-Br the gel was poured on tray and left for polymerization. The gel in tray was embedded 1X TAE Buffer in the

electrophoresis tank. Into the PCR products 10µl of 6X DNA loading buffer was added and 10 µl of the samples was loaded in the wells. The samples were run at 80V for 40 minutes and gels were analyzed under BioRads transilluminator and photographs of the gels were taken using the software MultiAnalyst of BioRad.

3.14 SEQUENCING OF DNA FRAGMENTS

3.14.1 ISOLATION OF DNA FRAGMENTS

PCR product was run in 1% agarose gel. The band corresponding to subject fragment was spliced out the gel using a clean blade and put in 1.5 ml eppendorf tube. The weight of the slice was measured. Isolation of the DNA from the gel was carried out using the MN's Gel-DNA isolation kit as described in the manual of the kit.

3.14.2 PCR

Using the DNA purified from the gel as template a new PCR was carried out using the conditions appropriate for the fragment of interest.

3.14.3 TA-CLONING

Promega's TA-cloning kit was used to clone fragments produced by PCR. Vector used was pGEM-T. The reaction mixture contained 50ng of vector DNA, 100ng of insert DNA, 7.5µl of 2X ligase buffer, and 3 units of T4 ligase supplied in the kit. The mixture was incubated at room temperature for 2 hours and supercompetent DH5-α bacterial cells were transformed using the whole ligation product. Cells were spread on LB-agar plates containing ampicilline, IPTG and X-gal. Plates were incubated in 37˚C incubator for 16 hours. White colonies were selected as candidate clones and inoculated in fresh liquid LB medium containing ampicilline.

3.14.4 SCREENING OF COLONIES

Candidate clones were grown overnight and from the cultures plasmid DNA was isolated using the manual mini-prep protocol as in section 3.3. 5µl DNA samples of each clone were cut using appropriate restriction enzymes in appropriate buffers. The restriction was carried out in a total volume of 20µl containing 2µl 10X reaction buffer, 5µl DNA, 1µl specific restriction enzyme (1 units/µl) , and ddH_2O. Mixture was incubated in 37˚C water bath for 2 hours and whole restriction product was run on 1% agarose gel.

3.14.5 ISOLATION OF PLASMID DNA

For automated sequencing highly pure DNA is needed and for this purpose the plasmid DNA isolation from candidate clones was done using MN's plasmid DNA isolation mini-prep kit as described in the manual. Mini-prep products were run on agarose gel using a mass ruler to have an idea about the concentration of the band. (Important for automated sequencing reactions)

3.14.6 AUTOMATED SEQUENCING

Automated sequencing was carried out by the technicians in our lab using the kit. For each sequence reaction appropriate primers were diluted at least to a 1 pico molar concentration.

CHAPTER 4

RESULTS

4.1 SUMMARY OF RESULTS

Effect of E2F1 overexpression on p73 transactivation was demonstrated by transient transfections followed by RT-PCR. For transfections plasmid DNA and constructs were isolated from bacterial cells with maxi-prep. Concentrations of plasmids were analyzed with spectrophotometry and compared with agarose gel electrophoresis (**section 4.2**). 6 different cell lines were transfected with E2F1 construct together with pEGFP-N2 as an efficiency control. E2F4 transfections and untransfected cells were used as negative controls. RNA and cDNA were prepared from cells 24 hour after transfection. Templates were equalized with GAPDH PCR (**Section 4.4.2**). As an indirect control of E2F1 expression $p14^{ARF}$ PCR was done with HepG2 cell line samples (**Section 4.4.3**). Then PCRs were done with C-terminal, TA-p73, and DN-p73 primers (**Sections 4.4.4, 4.4.5, 4.4.6** respectively).

Time course activation of p73 isoforms were demonstrated on Cama-1 cells with transient transfection, followed by RNA isolation, cDNA synthesis and p73 PCRs at 4 hour intervals. E2F1, TA-p73 and wt p53 transfections were done. TA-p73 transfection was done to see whether DN-p73 was transactivated in response to TA-p73. Wild type p53 was used as a positive control of p73 gene transactivation; because it is known that it transactivates both TA-p73 and DN-p73 promoters (Kartasheva et al 2002,). GAPDH equalization, TA-p73 and DN-p73 PCRs were done using cDNAs provided (**sections 4.5.2, 4.5.3, 4.5.4** respectively).

The bands obtained from PCRs of TA-p73 were sequenced (**section 4.6**).

4.2 PRODUCTION OF PLASMIDS AND CONSTRUCTS

pEGFP-N2, pCDNA3-TA-p73, pRC/CMV-E2F1, pRC/CMV-E2F4, pRC/CMV-p53 vectors and constructs were transformed into super-competent DH5-α bacterial cells. Transformed cells were grown overnight in 250 ml LB with appropriate antibiotic. Using Qiagen' s Maxi-Prep Kit, plasmid DNA from these cultures were isolated as described in the kit's manual. 3µl from each sample were run on 1% agarose gel and concentrations of the plasmid solutions were detected with spectrophotometric measurement.

Table 4: Spectrophotometric measurements of plasmid DNA samples

Sample	Net Abs 260	Net Abs 280	260/280	Dilution fa	Concentration (ug/ml)
pCDNA3.1c	0.526	0.3036	1.73	100.00	2630.00
pEGFP-N2	0.4309	0.2542	1.70	100.00	2154.57
pCDNA3-TAp73	0.2466	0.1379	1.79	100.00	1233.10
pCMV-E2F1	0.3407	0.2038	1.67	100.00	1703.40
pCMV-E2F4	0.3524	0.2072	1.70	100.00	1704.99
pCMV-p53	0.2119	0.1175	1.80	100.00	1059.5

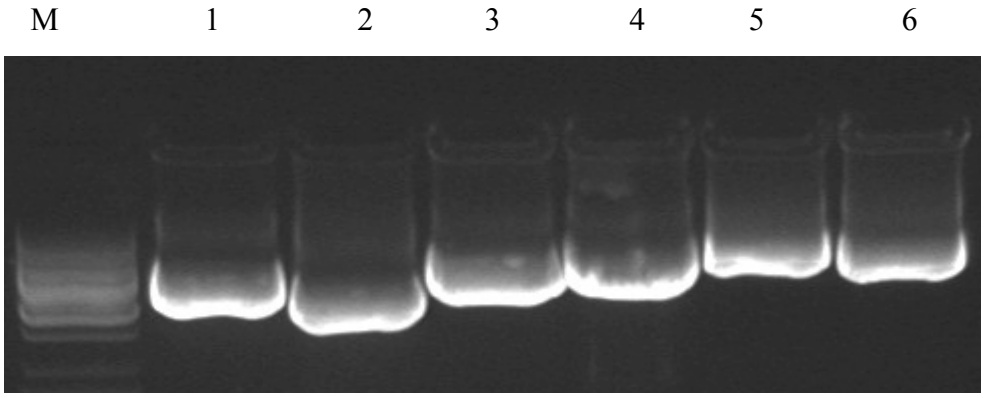


Figure 7: Agarose gel electrophoresis of Maxi-prep products in 1% gel. 5µl is loaded from each sample.

Lanes:

M) Marker

1) pCDNA3.1.C, 2) pEGFP-N2, 3) pRC/CMV-p53, 4) pCDNA3-p73,

5) pRC/CMV-E2F1, 6) pRC/CMV-E2F4

4.3 OPTIMIZATION OF TRANSFECTION

Saos-2, CAMA1, Hep3B and HepG2 cell lines grown in 60mm plates were transfected with 1µg, 2µg, 5µg, 10µg and 30µg pEGFP-N2 vector. 16 hours post-transfection medium of the cells were changed. 24 hour post-transfection transfection efficiencies were observed under inverted fluorescence microscope.

It was seen that 30 µg DNA has the highest efficiency at each cell line and did not harm the cells. In CAMA1 efficiency was over 50%. In Saos-2, Hep3B and HepG2 efficiencies were 20%, 25% and 25% respectively.

4.4 ECTOPIC EXPRESSION OF E2F1 AND E2F4 AND THEIR EFFECT ON TRANSCRIPTION OF P73 GENE.

Saos2, Cama1, Hep3B, HepG2, SK-Hep1, and SNU398 cells grown in 100mm plates were transfected together with 20µg E2F1 or E2F4 and 10µg of pEGFP-N2. Extra plates of cells, which were not transfected, were grown as negative control. Transfection efficiencies were as observed in optimization experiment. For SK-Hep1 and SNU398 efficiency were lower than other cell lines (20% and 10% respectively)

14 hour post-transfection the media of cells were refreshed. 24 hour post transfection cells were scraped in RA1 buffer of MN's nucleospin RNA isolation kit and RNA isolation was done as described in the manual of the kit. Spectrophotometric and gel electrophoresis analysis of the RNA samples were done.

4.4.1 cDNA SYNTHESIS

3µg of RNA from each sample was used in cDNA synthesis. cDNA synthesis was done using MBI's RevertAid first strand cDNA synthesis kit as described in the manual. Each cDNA product (20µl) was diluted in 2 volumes (40µl) deionized water supplied with the kit.

4.4.2 GAPDH PCR OF THE cDNA SAMPLES:

As a control of equal loading, a gene abundantly expressed in all cells is used. Here GAPDH gene was used. 2µl from each cDNA sample was used as template and PCR reactions were done as described in methods.

	Saos-2			Cama-1			Hep3B			HepG2			SK-Hep1			SNU398			
E2F1	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	
E2F4	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	-ve

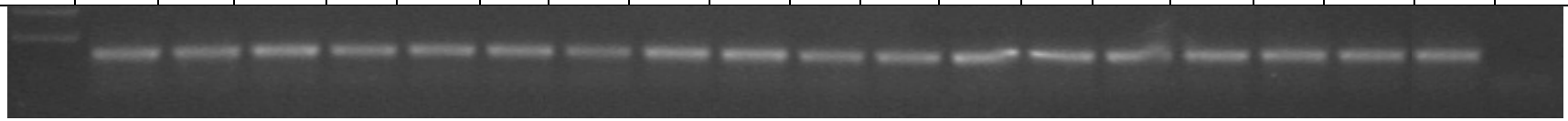


Figure 8: GAPDH PCR. Using GAPDH PCR, templates were equalized for further semi-quantitative PCR reactions.

It is important to use equal amounts of cDNA as template in semi-quantitative PCR. Using GAPDH PCR the template amount for further PCR reactions is equalized.

4.4.3 P14^{ARF} PCR FOR CHECKING THE EXPRESSION OF E2F1

p14^{ARF} is one of the target genes transactivated by E2F1, so we used it as a control of E2F1 expression. Using 2µl from HepG2 samples as template p14^{ARF} PCR were done in 50µl reaction volume. Conditions were as described in the methods. Each PCR product was mixed with 10µl 6X DNA loading buffer and run on 1% agarose gel.

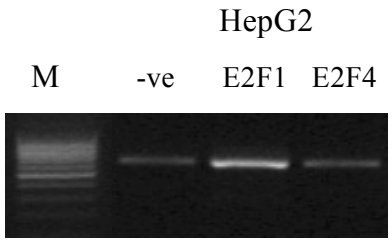


Figure 9: p14 ARF PCR of HepG2 samples.

Figure 9 shows that the cells have ectopic expression of E2F1. Increase in the amount of p14 transcript is due to the transcriptional activation of p14 promoter by the expression of E2F1 gene exogenously inserted into the cells.

4.4.4 PCR FOR p73 C-TERMINAL VARIANTS

7 µl from each cDNA sample was used in PCR in 50µl reaction volume using conditions described in methods. 6 µl of PCR products were run on 1% agarose gel after mixing them with 10µl of 6X DNA loading buffer.

	Saos-2			Cama-1			Hep3B			HepG2			SK-Hep1			SNU398			
E2F1	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	
E2F4	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	-ve

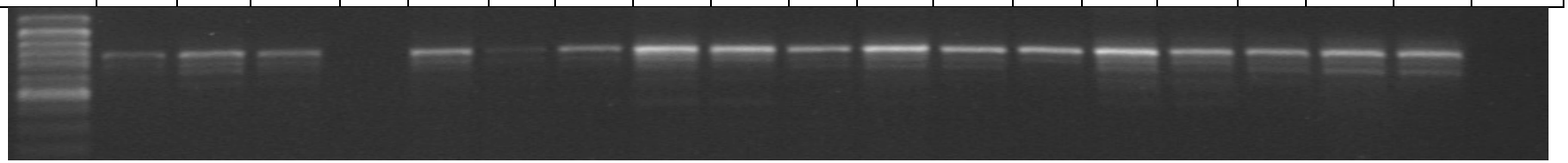


Figure 10: PCR of samples for detection of C-Terminal Variants.

It is clear that expression of all C-terminal variants (there are six variants identified) are induced in response to E2F1 overexpression. E2F4 induces the expression of p73 relative to the untransfected controls but it is not as much as E2F1 does. The predominant form seems to be the alpha p73 in all cell lines.

4.4.5 PCR FOR TA-p73

7 µl from each cDNA sample was used in PCR in 50µl reaction volume using conditions for described in methods. 10 µl of PCR products were run on 1% agarose gel after mixing them with 10µl of 6X DNA loading buffer.

	Saos-2			Cama-1			Hep3B			HepG2			SK-Hep1			SNU398			
E2F1	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	
E2F4	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	-ve

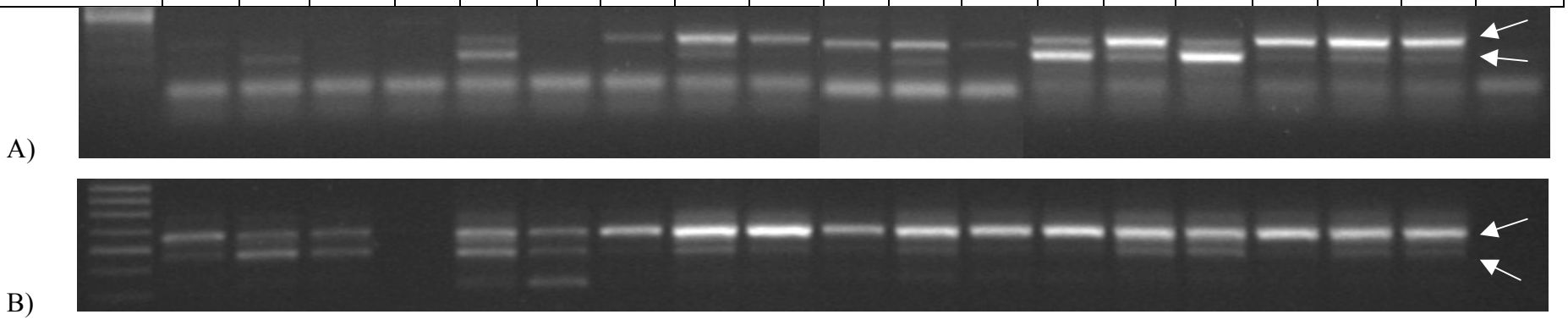


Figure 11: Transactivation of TA-p73 and two other N-terminal splice variants in response to E2F1 over-expression

Rows:

A) PCR using 1st set of TA-p73 primers (Fillipovich et al 2001)

B) PCR using 2nd set of TA-p73 primers.

Figure 11-A and B are demonstrations of induction of TA-p73 and p73-Δexon2 forms in response to E2F1 overexpression. The upper band in figure 11-A is TA-p73. However lower band was shown to be E2F1 instead of p73-Δexon2 form. In order to discard this

parasite band we repeated the PCR reactions with new primer set in Figure 11-B. The two bands in B-panel was shown to be TA-p73 and p73-Δexon2 forms. The induction level of E2F1 is higher than E2F4 and untransfected controls. E2F4 has also a level of induction higher than untransfected cells. The basal level of p73 forms differ in different cell lines. For example Cama-1 seem to have no p73 transcripts. Induction of E2F1 seem to induce both TA-p73 and p73-Δexon2 forms at equal amounts. On the other hand HCC cell lines seem to express predominantly TA-p73 form. However expression of E2F1 induce both TA-p73 and p73-Δexon2.

4.4.6 PCR FOR DN-p73

7 µl from each cDNA sample was used in PCR in 50µl reaction volume using conditions described in methods. 10 µl of PCR products were run on 1% agarose gel after mixing them with 10µl of 6X DNA loading buffer.

	Saos-2			Cama-1			Hep3B			HepG2			SK-Hep1			SNU398			
E2F1	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	
E2F4	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	-ve

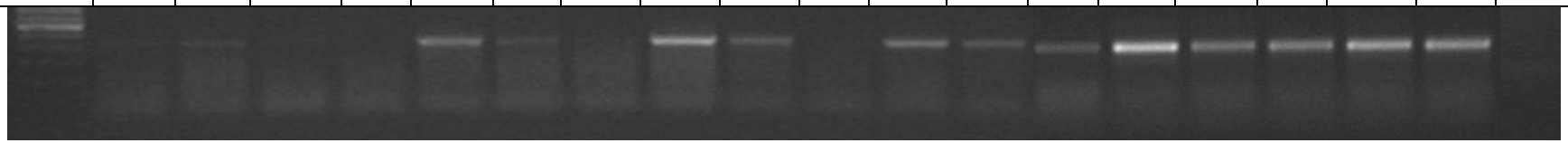


Figure 12: DN-p73 PCR.

E2F1 transactivates DN-p73 form next to TA-p73 and p73-Δexon2 forms. Similar to TA-p73 PCR, there is basal expression of DN-p73 form in HCC cell lines. Again E2F4 induced a level of DN-p73 relative to untransfected controls. E2F1 expression caused the highest level of induction in DN-p73 form in SK-Hep1 cell line.

If summarized, it is absolute that E2F1 induces the expression of at least three p73 forms, two of which are dominant negative forms. However the basal levels and extent of induction of different p73 isoforms seem to be cell type specific.

Table 5: Summary of sections 4.4.3-4.4.6; Induction of TA-p73, p73-Δexon2 and DN-p73 forms in response to E2F1 overexpression.

Cell		Status of Endogenous				Untransfected			E2F1 overexpression			E2F4 overexpression		
Line	**Origin**	**53**	**pRb**	**p16**	**p14**	**TA-p73**	**p73-Δex2**	**DN-p73**	**TA-p73**	**p73-Δex2**	**DN-p73**	**TA-p73**	**p73-Δex2**	**DN-p73**
Saos-2	Osteosarc.	-ve	-ve	+	+	++	+	-ve	+	++	+	+	+	+
CAMA-1	Breast CA	MT	+	-ve	+	-ve	-ve	-ve	++	++	++	+	+	±
Hep3B	HCC	-ve	-ve	+	+	++	±	-ve	++++	+	++	+++	±	+
HepG2	HCC	WT	+	+	+	++	±	-ve	+++	+	++	++	±	+
SK-Hep1	HCC	WT	+	-ve	-ve	+++	±	+	++++	+	+++	+++±	+	++
SNU398	HCC	-ve	+	weak	+	+++	±	+ +	+++±	+	+++	+++±	+	++

Highlighted parts indicate that there is an increase.

4.5 TIME-COURSE ACTIVATION OF P73 SPLICE VARIANTS

According to RT-PCR data done 24 hour post-transfection, it seems to be both TA and DN forms of p73 are activated. However it may be biologically important which form is activated first. If TA-p73 form is activated, then the activation of DN-p73 form maybe a consequence of TA-p73 activation, because it is known that one of the targets of activated TA-p73 protein is the DN-p73 promoter. On the other hand DN form can be a direct target of E2F1 transcription factor and may be activated simultaneously or DN-p73 form may be activated before TA-p73 form is activated. For this purpose we prepared 3 sets of cells for p53 transfection, p73 transfection and E2F1 transfection. For each set 8 plates of Cama-1 cells were splitted 24 hour before transfection (250.000 cells per plate). These 8 plates were for harvesting cells post-transfection with different time intervals (4hours, 8hours, 12hours, 16hours, 20hours, 24 hours, 36 hours, and 60 hours). Transfections were done as described methods using 20µg of pRC/CMV-p53 or pCDNA3-p73 or pRC/CMV-E2F1 together with 10µg of pEGFP-N2 as a efficiency control. Efficiency of transfection was same in all plates (~60%). After 4hours, 8hours, 12hours, 16hours, 20hours, 24 hours, 36 hours, and 60 hours post-transfection, at each time one plate from each three set was taken and the media were aspirated. Cells were washed with cold PBS for 3 times and scraped with 350µl of RA1 buffer of MN's RNA isolation kit. RNA isolation was done using the kit as described in the manual. Concentration of the isolated RNA samples were measured using spectrophotometry with a dilution of 100 in DEPC treated ddH_2O. RNA samples were run on agarose gel.

4.5.1 cDNA SYNTHESIS

Unfortunately some samples were not enough concentrated to do the synthesis reactions with 3µg of RNA. So this time 2µg RNA was used in cDNA synthesis reactions. Synthesis was carried on as described in the MBI's RevertAid first strand cDNA synthesis kit manual.

4.5.2 GAPDH PCR OF THE cDNA SAMPLES:

3.5µl from each cDNA sample was used as template in PCR reactions done as described in methods. 10 µl of PCR products were run on 1% agarose gel after mixing them with 10µl of 6X DNA loading buffer.

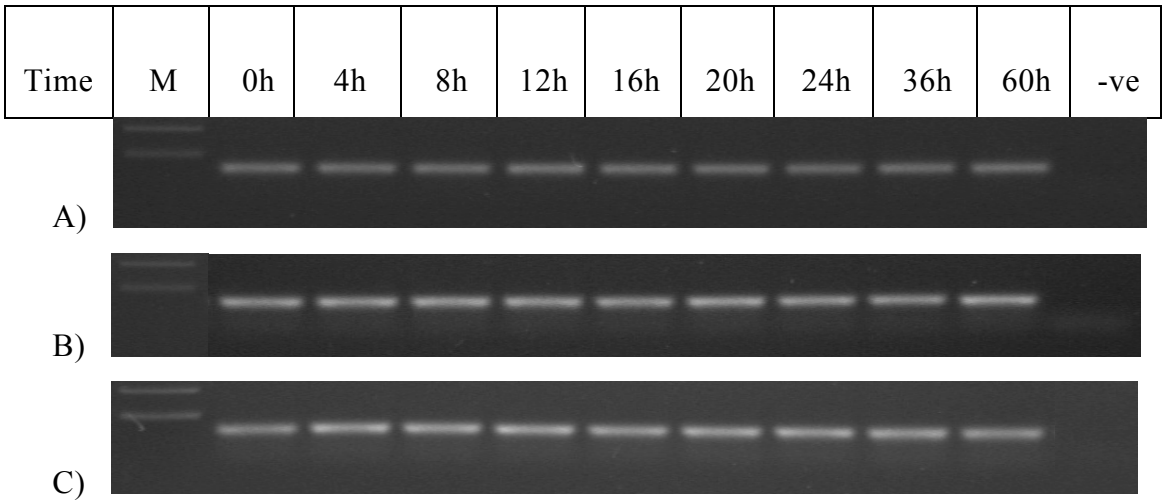


Figure 13: GAPDH PCR of E2F1 (Row A), wt p53 (Row B), and TA-p73 (Row C) sets were used to equalize the template amounts for following PCR reactions.

4.5.3 TA-p73 PCR

Samples provided by the wt p53 and E2F1 transfections were used for TA-p73 PCR. From each sample 11µl of cDNA was used as template in the PCR reactions done as described in methods. Second set of TA-p73 primers were used. 10 µl of PCR products were run on 1% agarose gel after mixing them with 10µl of 6X DNA loading buffer.

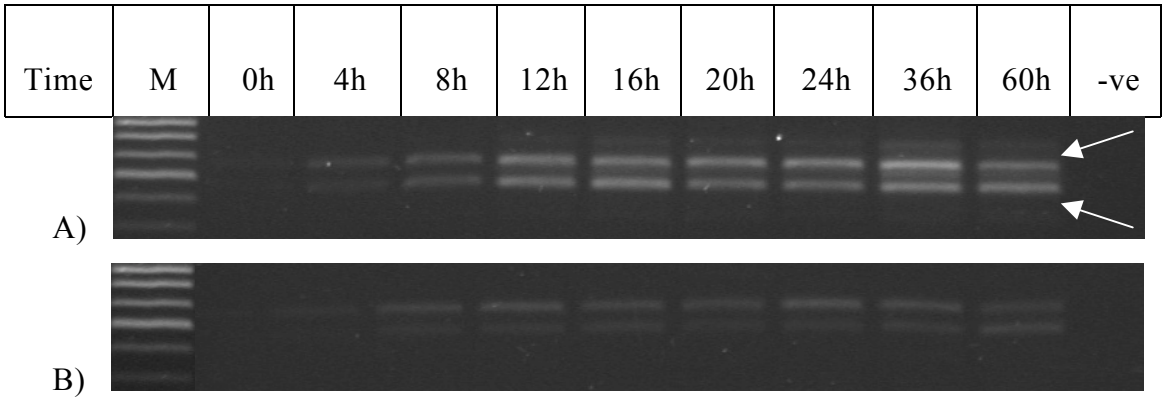


Figure 14: Time course transactivation of TA-p73 and p73-Δexon2 splice variants of p73 in response to E2F1 (Row A) and wt p53 (Row B) over-expression.

Arrows in **figure 14-A** show the TA-p73 and p73-Δexon2 forms (upper and lower bands respectively). It is clear that there is a time dependent increase in transcription of both forms in response to E2F1 overexpression. The transcripts can be detected at 4^{th} hour. Induction makes a peak at 12^{th} hour and a second peak at 36^{th} hour. A similar pattern with a lower intensity of bands can be seen in wt p53 overexpression instead of E2F1 (**Figure 14-B**). Effect of TA-p73 was not tested due to technical problems.

4.5.4 DN-p73 PCR

Samples of all 3 sets (E2F1, TA-p73 and wt p53 transfections) were tested for activation of DN-p73 using 11µl of cDNA as template. Conditions were as described in methods. Products were run on 1% agarose gel.

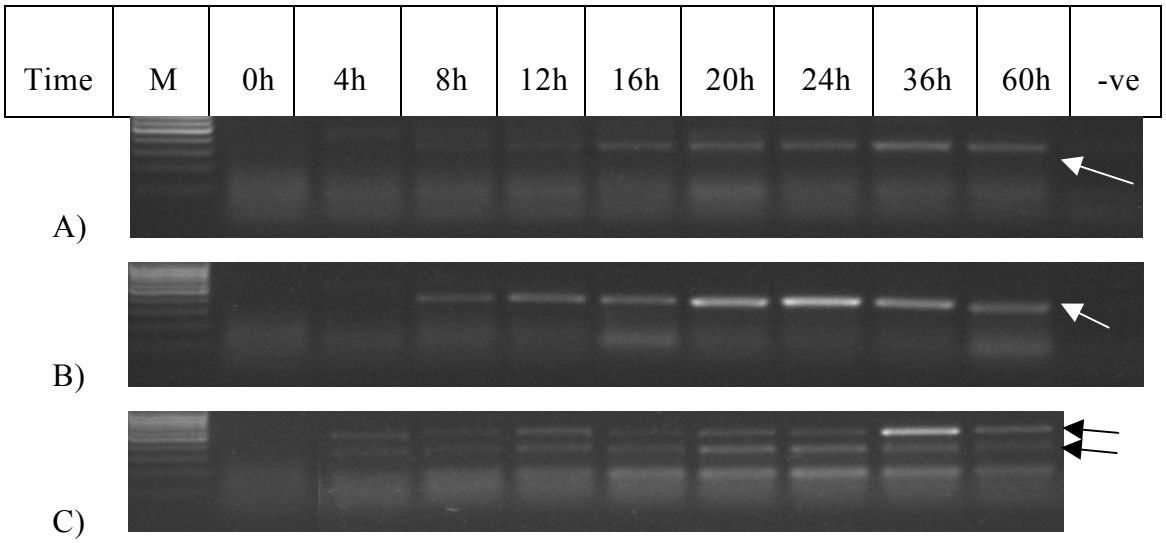


Figure 15: Time-course transactivation of DN-p73 splice variant in response to E2F1 (Row A), wt p53 (Row B), and TA-p73 (Row C) over-expression in Cama1 cells. Arrow shows a specific cross-reacting band, which is most probably TA-p73.

Arrows in Figure 15-A and B shows the DN-p73 bands induced in response to E2F1 and wt p53 overexpression respectively. DN-p73 transcripts are detectable at 8th hour after E2F1 expression. The pattern in wt p53 overexpression is same but the intensity of bands is significantly higher than E2F1. DN-p73 induction makes peak at 36th and 24th hours after E2F1 and wt p53 expression respectively. TA-p73 also induces transcription of DN-p73 (lower bands with arrow in Figure 15-C). However DN-p73 primers specifically cross-reacted with ectopic TA-p73 that there are parasite bands (upper bands with arrow in figure 15-C) However it can be seen that DN-p73 is induced in response to TA-p73at 4th hour.

As a summary Figures 14 and 15 demonstrates the time-course activation of TA-p73, p73-Δexon2, and DN-p73 in response to E2F1, wt p53 and TA-p73 overexpression. DN-p73 is induced with four hours later than TA-p73 is induced in response to E2F1. TA-p73 and p73-Δexon2 forms are induced together and at

equal amounts. Wt p53 is capable of inducing both TA-p73 and DN-p73. TA-p73 induces DN-p73 induction.

Table 6: Time course activation of TA-p73, p73-Δexon2 and DN-p73 forms in response to E2F1, wt p53 and TA-p73 overexpression.

		TRANSFECTIONS		
		E2F1	**wt p53**	**TA-p73**
TA-p73	0h	±	-ve	N.D.
	4h	+	±	N.D.
	8h	++	+	N.D.
	12h	+++	+±	N.D.
	16h	+++	+±	N.D.
	20h	+++	+±	N.D.
	24h	+++	++	N.D.
	36h	++++	+±	N.D.
	60h	++	+	N.D.
	Start of induction	4th hour	4th hour	N.D.
	Peak at	12th hour, 36th hour	24th hour	N.D.
p73-Deltaexon2	0h	±	-ve	N.D.
	4h	+	±	N.D.
	8h	++	+	N.D.
	12h	+++	+	N.D.
	16h	+++	+	N.D.
	20h	+++	+	N.D.
	24h	+++	+	N.D.
	36h	+++	+	N.D.
	60h	++	+	N.D.
	Start of induction	4th hour	4th hour	N.D.
	Peak at	12th hour	16th hour	N.D.
DN-p73	0h	-ve	-ve	-ve
	4h	-ve	-ve	±
	8h	±	±	±
	12h	±	+	+
	16h	+	+	±
	20h	+	++	++
	24h	+	+++	++
	36h	++	++	+
	60h	+	+	+
	Start of induction	12th hour	8th hour	4th hour
	Peak at	36th hour	24th hour	20th hour

4.6 SEQUENCING OF THE BANDS PROVIDED BY THE PCR REACTIONS

After the PCR products were run, the bands for sequencing were spliced out and purified. A second PCR was done with these purified products using same primers. For sequencing either the purified PCR product or the constructs made by cloning these PCR products into pGEM-T vectors were used.

The extra band amplified by 1st set of TA-p73 primer was found to be E2F1, not Δexon2 spliced form of p73 as we thought (Figure 11, Row A, lower band). Surely this does not mean that there was not any amplified Δexon2 spliced form of p73. The amplified E2F1 parasite band had exactly the same length that it should have masked the p73Deltaexon2 spliced form either on the gel or in PCR reaction by competition. With a new set of TA-p73 primers the PCR reactions were repeated (Figure 11, Row B) and the new bands were sequenced. They were confirmed to be TA-p73 and p73-Δexon2 forms.

CHAPTER 5
DISCUSSION

DISCUSSION

Our data demonstrate that in all cell lines used, transcription of at least three N-terminal variants of p73 is induced in response to E2F1 over-expression. E2F1 was successfully expressed in cells after transient transfection, as demonstrated by $p14^{ARF}$ PCR **(Figure 9**). **Figure 10** shows that all C-terminal variants of p73 were induced; being the alpha form the abundant one. E2F4 expression caused also a degree of increase in p73 transcripts but, it was not as dramatic as E2F1 did. First set of TA-p73 primers (Fillippovich et al 2001) showed increase in two bands corresponding to TA-p73 and p73-Δexon2 forms in response to E2F1 expression (higher and lover bands respectively) (**Figure 10**). However, sequencing analysis revealed that this band was E2F1. Interestingly the primers have more than 50% homology to different regions on E2F1, resulting in parasite bands, which should have masked the p73-Δexon2 form by either competing in PCR or blocking its view on the gel. In order to avoid parasite bands we designed another primer set which could detect three forms of 73; TA-, Δexon2, and Δexon2-3 forms. Semi-quantitative PCR with these primers revealed similar results as first set of primers. It was also shown that obtained bands were TA-p73 and p73-Δexon2 forms with sequence analysis. Expression of both TA-p73 and p73-Δexon2 forms were induced in response to E2F1 with respect to untransfected and E2F4 transfected controls. Interestingly there was another band smaller than two forms, which maybe the p73-Δexon2-3 form induced in both E2F1 and E2F4 transfections of Cama-1 cells (**Figure 11, Row B, Cama-1 lanes**). In addition, basal and E2F1 induced levels of TA-p73 and p73-Δexon2 expressions were different in different cell lines. HCC cell lines have predominantly TA-p73 form. There was not a basal expression in Cama-1 and E2F1 caused expression of equal amounts of TA-p73 and p73-Δexon2 transcripts.

It is very clear that DN-p73 is transactivated in all cell lines with E2F1 over-expression **(Figure 12**). Similar to TA-p73, different cell lines have different basal expression levels of DN-p73 form. HCC cell lines had basal expression of DN-p73, whereas untransfected Cama-1 was negative of DN-p73 expression. E2F1 expression resulted in induction of transcription of DN-p73,

which was not demonstrated before. E2F4 caused an increase in Cama-1, HepG2, and Hep3B less than E2F1. There is a possibility that this DN-p73 transactivation was not a direct effect of E2F1. It was shown before that DN-p73was transactivated by TA-p73 and induction of DN-p73 in our data may be a consequence of TA-p73 transactivated by E2F1. A semi-quantitative RT-PCR after cycloheximid treatment of E2F1 transfected cells may reveal whether DN-p73 is a direct target of E2F1.

For determination of cell fate, whether proliferation, cell cycle arrest or apoptosis will be induced the timing of proliferative and anti-proliferative signals is very important. The way the cell will be forced may be different depending on the form of p73 activated first. In a time-course transactivation experiment, we demonstrated the transactivation of DN-p73, TA-p73 and p73-Delta-exon2 forms in 4 hour intervals, in order to see the sequential activation of different forms. As controls we used wt p53 and TA-p73 transfections. E2F1 expression resulted in a continuous increase in the transactivation of both TA-p73 and p73-Δexon2 forms starting from 4^{th} hour, and making a peak at 36^{th} hour (**Figure 14. Row A**). Interestingly the band intensities of two forms seem to be equal, and this may mean that nearly equal amounts of TA-p73 and p73-Δexon2 proteins are induced by E2F1 in cells, neutralizing each other. Transactivation of DN-p73 seems to be activated at 8^{th} our after E2F1 transfection (**Figure 15, Row A**). TA-p73 and wt p53 could transactivate DN-p73 starting at 8^{th} and 4^{th} hours respectively (**Figure 15, Rows B and C**). Effect of TA-p73 on DN-p73 expression strengthens the possibility that DN-p73 activation in response to E2F1 might be mediated by TA-p73, however it should be tested.

Actually if there was not an activation of p73-Δexon2 form, we could conclude that 4 hour earlier activation of TA-p73 than DN-p73 form could guide the cell to apoptosis or cell cycle arrest. However transactivation of equal amount of p73-Δexon2 form maybe be the early inhibitor of TA-p73 before DN-p73 form is transactivated. If TA-p73 is inhibited by p73-Δexon2, activation of DN-p73 transcription might be a direct effect of E2F1 or indirect effect of E2F1 using other mediators rather than TA-p73.

Our wt p53 controls showed that TA-p73, p73-Δexon2 and DN-p73 forms were transactivated in response to wt p53 over-expression (**Figure 14 row B,**

Figure 15 row B respectively). When compared with E2F1 transfection (**Figure 14 row A**), wt p53 induced transactivation of TA-p73 weaker than E2F1 did (**Figure 14 row B**). However transactivation of DN-p73 by wt p53 was stronger than E2F1 (**Figure 15 rows A, B**) confirming a recent data about targeting of wt p53 to DN-p73 promoter (Kartasheva et al 2002).

E2F1 protein has proliferative and anti-proliferative functions. This seem to be one of the paradoxes not yet have been solved. There are other molecules seem to have dual functions such as Myc and E1A. E2F1 was shown to induce activation genes involved in proliferation (cell cycle regulation, DNA replication) and apoptosis. Correlating with activation of this variety of genes, E2F1 was shown to induce either proliferation or apoptosis in different systems. It was shown that it induced S-phase entry and DNA synthesis (Johnson et al 1993; Vigo et al 1999). G_1 phase blocks induced by pRb, CKIs, TGF-Beta, and gamma-irradiation were overcome by E2F1 overexpression (Zhu et al 1993; DeGregori et al 1995; Lucas et al 1996; Schwarz et al 1995). Together with *Ras* activation E2F1 deregulation transformed primary Rat fibroblast cells (Johnson et al 1994). In different conditions E2F1 overexpression induced apoptosis (Wu et al 1994, Qin et al 1994; Kowalik et al 1994; Lissy et al 2000; Stiewe and Putzer 2000; Irwin et al 2000). Both positive and negative effects on tumor development were seen in response to absence or presence of E2F1 (Johnson 2000). Similarly p73 gene encodes different proteins working as antagonist of each other. While TA-p73 transactivates apoptotic genes, DN-p73 inhibits TA-p73 via hetero-oligomerization. Consequently p73 gene seems to encode both a tumor-suppressor and an oncogene.

There are models proposed to solve the paradox of activation of both proliferative and apoptotic signals by the same factor. Previously it was proposed that the induction of apoptosis by proliferative factors was a reaction of cell to the unscheduled and abnormal cell cycle progression. This model depends on the activation of apoptosis by conflicting growth signals. However another model suggests that induction of apoptosis is a normal consequence of cell cycle progression (Harrington et al 1994). This model couples the signals inducing cell cycle and apoptosis. Stimulation of cells to enter cell cycle promotes apoptosis at the same time. However the fate of the cell is determined later by additional apoptotic and proliferative signals. This model is supported by experiments on

different systems, in which change of same parameter led to both apoptosis and proliferation, most probably because of different sets of factors in these different systems deciding the fate of the cell in addition to the first stimulating factor. Correlating with this model, E2F1 activated both apoptotic and antagonist p73 forms in our experiments. Interestingly the basal levels of these forms were different in different cell lines demonstrating that there are other factors deciding the final fate of cell in addition to the E2F1 itself. The activation extent of activating and dominant negative forms in response to E2F1 was different in different cell lines. For example TA-p73 form was abundantly expressed in HCC cell lines and E2F1 induction increased TA-p73 more than other forms. Although dominant negative forms were increased too, they were not as much as TA-p73 form. This means abundant TA-p73 may induce activation of apoptosis despite the presence of dominant negative forms, which are present in lower amounts. However, still other factors such as presence of mutant p53 may inhibit TA-p73 (Di Como et al 1999). On the other hand, Cama-1 cells had no p73 transcript in untransfected state. E2F1 induction led to a similar amount of increase in TA-p73 and p73-Δexon2 forms. In addition, DN-p73 form was induced. Total amount of dominant negative form transcripts were higher than TA-p73 forms. In these cells activation of p53 and TA-p73 induced apoptosis or cell cycle arrest by E2F1 may be inhibited by the dominant negative forms.

Activation of both proliferative and apoptotic pathways by the same factor give the cell the chance to select one of the ways easily depending on the status of the cell and other factors found in the cell. If the cell is not ready for a following division, the cells may enter cell cycle arrest and provide time for cells to check for problems and get ready. If the cells can get ready and repair themselves in this time the cells are committed to enter cell cycle by extra factors and the commitment of apoptosis is cancelled with activation of additional anti-apoptotic signals. If there are problems those can not be recovered cells are led to apoptosis. Such a scenario is supported with our data demonstrating that the different forms of p73 were sequentially activated in response to E2F1 induction. Time-course induction of E2F1 induced DN-p73 form 8 hours later than it induced TA-p73 from. TA-p73 form may cause a cell cycle arrest in cells to provide time for cells to get ready and decide whether to go division or apoptosis. If the cells are ready for division activation of DN-p73 eight hours later, helps the

cells to recover cell cycle arrest caused by TA-p73 and additional factors induce cell cycle progression. If the cell decides to go apoptosis additional apoptotic signals are activated and the effect of DN-p73 activated later is diminished, leading cells to apoptosis.

It is necessary to think about the other pathways induced by E2F1 overexpression. Being a transcription factor there are many genes seem to be a target of E2F1. It can be a big mistake thinking activation of p73 gene independent of other pathways. P53 was shown to be directly and indirectly activated by E2F1. The indirect effect is mediated by $p14^{ARF}$, which prevents MDM2 to target p53 to proteolysis. Direct effect of E2F1 on p53 was shown to be by direct protein-protein binding, in the absence of cyclin A. This binding stabilized p53 and induced apoptosis (Hsiesh et al 2002). In addition to stabilization and activation of p53 the activation of dominant negative p73 forms by E2F1, maybe a safeguard mechanism to balance the apoptotic and proliferative signals for the sake of cell, as it was shown before that p53 was a target of dominant negative p73 forms (Pozniak et al 2000).

The status of the p53 in the cells is also important in determination of cell fate in response to activation of p73 forms with E2F1 overexpression. Several mutant forms of p53 are capable of blocking TA-p73 (Di Como et al 1999). Presence of such mutants in cells may act as inhibitors of TA-p73 induced apoptosis.

In cancer development deregulation of such dual mechanisms may play important roles. Induction of both proliferative and anti-proliferative signals at the same time by the same factor may be advantageous to cells to develop cancer if somehow they discard the apoptotic signals despite the abnormalities in the cell. The presence of acquired expression of TA-p73 gene in cancer cells may be a consequence of such a dysregulation (Sayan et al 2001). These cells are resistant to apoptosis although they seem to have excess amount of TA-p73 transcripts which are not present in normal tissues the cancer cells are derived from. Expression of dominant negative forms in addition to TA-p73 form in response to oncogenic activation may be the fact that these cancer cells tolerate TA-p73 mediated apoptosis. Another mechanism can be the generation of p53 mutants which can inhibit TA-p73. Previous study by Sayan et al demonstrated such a correlation between p73 and mutant p53 (Sayan et al 2001). Our data may

suggest that expression of dominant negative forms may be another mechanism cancer cells use to prevent TA-p73 or wt p53 induced apoptosis.

Interestingly there are a few mutations of p73 gene in thousands different cancer samples analyzed, conflicting with the idea that p73 is a tumor suppressor. However, it is not abnormal that a gene encoding both proliferative and anti-proliferative or apoptotic and anti-apoptotic protein products, does not show mutations in cancers. Presence of dual roles may give advantages to cancer cells. Such genes maybe more useful if not mutated for cancer cells. Similarly p73 gene which is not mutated and capable of encoding anti-apoptotic products may be more advantageous than a mutated one.

CHAPTER 6

CONCLUSION AND FUTURE PERSPECTIVES

6.1 CONCLUSION

Transactivating p73 was the only form shown to be transactivated by E2F1 overexpression previously. Consequence of TA-p73 activation in $p53^{-/-}$ was shown to be apoptosis in response to exogenous E2F1 expression (Lissy et al 2000; Irwin et al 2000; Stiewe and Putzer 2000). Confirming the previous data, we demonstrated the induction of TA-p73 in six different cell lines. The striking data we present is the induction of two extra forms of p73 by E2F1. Interestingly, these two forms, p73-Δexon2 and DN-p73 lack the transactivation domain of TA-p73 and so are called dominant negative forms. Their function is thought to be the inhibition of TA-p73 and p53 by hetero-oligomerization.

Although it was shown to induce apoptosis when overexpressed, E2F1 may cause different results in different cell lines depending on the sequential activation of different p73 forms. According to data obtained from time-course activation of p73 forms in response to E2F expression, DN-p73 form seem to be induced 4 hour later than TA-p73 and p73-Δexon2 forms. Presence of Δexon2 and DN-forms may inhibit the activity of TA-p73. Induction of cell cycle arrest or apoptotic genes by TA-p73 may be blocked by the dominant negative forms. In normal cells mostly, important effects for cell life are balanced with anti-effects. For example, p53 is activated in response to several stimuli including DNA-damage and induce apoptosis. Interestingly it also activates its negative regulator MDM2, which targets it to proteolysis. By this way such important signals kept transient and balanced well. If the cell can repair itself, apoptotic signal is cancelled. Sequential activation of p73 forms may have a similar role. In response to oncogenic activation such as E2F1, immediate response may be the TA-p73 transactivation, which may induce cell cycle arrest or apoptosis. After a certain time the antagonist of TA-p73, DN-p73 may be induced to balance the effect of TA-p73 and provide time for cells to repair themselves before entering apoptosis.

Such a dual p73 transactivation in response to oncogenic activation may be an advantageous strategy of cancer cells to overcome TA-p73. Actually it was shown before that cancer cells have an acquired TA-p73 expression (Sayan et al. 2001). However these cells were resistant to apoptosis, which should be activated

by prolonged TA-p73 activation. This resistance may be mediated by expression of dominant negative forms of p73 next to TA-p73 in response to oncogenic activation.

As a conclusion, dominant negative forms p73 are transactivated with TA-p73 in response to exogenous E2F1 expression, with an eight hour interval. Such a sequential activation may provide normal cells to respond oncogenic activation with cell cycle arrest and balance it with DN-p73 forms to give cells enough time for repair. Deregulation of this mechanism maybe advantageous for cancer cells to overcome apoptotic effects of prolonged TA-p73 activation.

6.2 FUTURE PERSPECTIVES

The most important question after demonstration of sequential activation of p73 forms in response to E2F1 expression is the consequences of this sequential activation for cells. We proposed that TA-p73 activated earlier than DN-p73 may induce cell cycle arrest, which may finally end with apoptosis. Later activation of DN-p73 may inhibit TA-p73 and provide time for cells to repair damages or to overcome oncogenic activation. In order to see this, the cells transfected with E2F1 should be observed in different time intervals to see whether they are arrested correlating with TA-p73 transactivation. The effect of later DN-p73 activation on cell cycle arrest may be seen whether DN-p73 weakens the effect of TA-p73 or not.

In order to distinguish the direct effect of E2F1 on DN-p73 promoter, a cycloheximid treatment can be done after E2F1 transfection and semi-quantitative PCRs for DN-p73 can be repeated. Cycloheximid is an inhibitor of translation. As far as the E2F1 protein is supplied the endogenous TA-p73 gene is transcribed but not translated, so if there is still induction of DN-p73 this means that E2F1 can directly activate DN-p73 promoter.

Mutant forms of p53 may be used to inhibit TA-p73 instead of cyloheximid treatment. E2F1 transfection of cell lines having mutant p53 or co-transfection of E2F1 with mutant p53 may be done and activation of DN-p73 may be tested with semi-quantitative RT-PCR

In order to demonstrate the activation of p73 in protein level, the western blot and immuno-fluorescence experiments can be done.

As a functional study, cell lines other than Saos-2, which respond TA-p73 transfection with apoptosis may be used to see the effect of E2F1 on apoptosis in different backgrounds. This may also show the effect of E2F1 on cell fate determination, when it transactivated different sets of p73 isoforms in different cell lines. A double staining of p73 and Annexin V or NAPO (Sayan BS et al 2001) can reveal in these cell lines whether p73 transactivation is co-localizing with apoptosis.

Construction of stable transformants of inducible E2F1 may be another choice to see the effect of E2F1 in 100% of cells, rather than transient transfections.

REFERENCES

Adams, P.D., Li, X., Sellers, W.R., Baker, K.B., Leng, X., Harper, J.W., Taya, Y., and Kaelin, W.G., Jr. "Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-CDK complexes." *Mol. Cell. Biol.* **19:** 1068-1080, 1999.

Adams, P.D., Sellers, W.R., Sharma, S.K., Wu, A.D., Nalin, C.M., and Kaelin, W.G., Jr. "Identification of a cyclin-CDK2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors." *Mol. Cell. Biol.* **16:** 6623-6633, 1996.

Agami R, Blandino G, Oren M, Shaul Y. " Interaction of c-Abl and p73α and their collaboration to induce apoptosis." *Nature*, vol:399, pp:809-813, 1999

Ahomadegbe JC, Tourpin S, Kaghad M, Zelek L, Vayssade M, Mathieu MC, Rochard F, Spielmann M, Tursz T, Caput D, Riou G, Benard J. "Loss of heterozygosity, allele silencing and decreased expression of p73 gene in breast cancers: prevalence of alterations in inflammatory breast cancers." *Oncogene*, vol:9;19, vol:5413-5418, 2000

Alonso ME, Bello MJ, Gonzalez-Gomez P, Lomas J, Arjona D, de Campos JM, Kusak ME, Sarasa JL, Isla A, Rey JA. "Mutation analysis of the p73 gene in nonastrocytic brain tumours." *Br J Cancer*., vol:85, pp:204-208, 2001

Alonso ME, Bello MJ, Lomas J, Gonzalez-Gomez P, Arjona D, De Campos JM, Gutierrez M, Isla A, Vaquero J, Rey JA. "Absence of mutation of the p73 gene in astrocytic neoplasms." *Int J Oncol*., vol:19, pp:609-612, 2001

Araki D, Uzawa K, Watanabe T, Shiiba M, Miyakawa A, Yokoe H, Tanzawa H. "Frequent allelic losses on the short arm of chromosome 1 and decreased expression of the p73 gene at 1p36.3 in squamous cell carcinoma of the oral cavity" *Int J Oncol*., vol;20, pp:355-360, 2002

Balint E, Bates S, Vousden KH. "Mdm2 binds p73 alpha without targeting degradation." *Oncogene*, vol:18, pp:3923-3929, 1999

Bandara, L. R., Buck, V. M., Zamanian, M., Johnston, L. H. & La Thangue, N. B. "Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F". *EMBO J.* **12**, 4317-4324, 1993.

Barrois M, Eychenne MK, Terrier-Lacombe MJ, Duarte N, Dubourg C, Douc-Rasy S, Chompret A, Khagad M, Hartmann O, Caput D, Benard J. "Genomic and allelic expression status of the p73 gene in human neuroblastoma" *Med Pediatr Oncol*., vol:36, pp:45-47, 2001

Bates, S. *et al*. "p14ARF links the tumour suppressors RB and p53". *Nature* **395**, 24-125 1998.

Beijersbergen, R. L. *et al*. "E2F-4, a new member of the E2F gene family, has

oncogenic activity and associates with p107 *in vivo"*. *Genes Dev.* **8**, 2680-2690, 1994.

Bookstein, R., Rio, P., Madreperla, S.A., Hong, F., Allred, C., Grizzle, W.E., and Lee, W.H. "Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma". *Proc. Natl. Acad. Sci.* **87:** 7762-7766, 1990.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. "Retinoblastoma protein recruits histone deacetylase to repress transcription". *Nature* **391:** 597-601, 1998.

Bremner, R., Cohen, B.L., Sopta, M., Hamel, P.A., Ingles, C.J., Gallie, B.L., and Phillips, R.A. "Direct transcriptional repression by pRb and its reversal by specific cyclins". *Mol. Cell. Biol.* **15:** 3256-3265, 1995.

Cai YC, Yang GY, Nie Y, Wang LD, Zhao X, Song YL, Seril DN, Liao J, Xing EP, Yang CS. "Molecular alterations of p73 in human esophageal squamous cell carcinomas: loss of heterozygosity occurs frequently; loss of imprinting and elevation of p73 expression may be related to defective p53." *Carcinogenesis*, vol:21, pp:683-689, 2000

Cartwright, P., Muller, H., Wagener, C., Holm, K. & Helin, K. "E2F-6: a novel member of the E2F family is an inhibitor of E2F-dependent transcription". *Oncogene* **17**, 611-623, 1998.

Chandar N, Billig B, McMaster J, Novak J. "Inactivation of p53 gene in human and murine osteosarcoma cells*"*. *Br J Cancer*, Vol:65(2) pp:208-14, 1992

Chen, P.L., Scully, P., Shew, J.Y., Wang, J.Y., and Lee, W.H. "Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation". *Cell* **58:** 1193-1198, 1989.

Chen, T.T. and Wang, J.Y. "Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function". *Mol. Cell Biol.* **20:** 5571-5580, 2000.

Chi SG, Chang SG, Lee SJ, Lee CH, Kim JI, Park JH. "Elevated and biallelic expression of p73 is associated with progression of human bladder cancer." *Cancer Res.*, vol:59, pp:2791-2793, 1999

Chow, K.N. and Dean, D.C. "Domains A and B in the Rb pocket interact to form a transcriptional repressor motif". *Mol. Cell. Biol*. 16: 4862-4868, 1996.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., and te Riele, H. "Requirement for a functional Rb-1 gene in murine development". *Nature* 359: 328-330, 1992

Claudio, P.P., Howard, C.M., Baldi, A., De Luca, A., Fu, Y., Condorelli, G., Sun, Y., Colburn, N., Calabretta, B., and Giordano, A. "p130/pRb2 has growth

suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107". *Cancer Res*. 54: 5556-5560, 1994.

Corbeil, H.B., Whyte, P., and Branton, P.E. "Characterization of transcription factor E2F complexes during muscle and neuronal differentiation". *Oncogene* 11: 909-920, 1995.

Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG. "Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation." *Cancer Res*., vol:59, pp:3352-3326, 1999

Cosma, M.P., Tanaka, T., and Nasmyth, K. "Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter". *Cell* 97: 299-311, 1999.

Dahiya, A., Gavin, M.R., Luo, R.X., and Dean, D.C. Role of the LXCXE binding site in Rb function. *Mol. Cell. Biol*. 20: 6799-6805, 2000.

Dalton, S. "Cell cycle regulation of the human CDC2 gene". *EMBO J.* 11, 1797-1804, 1992.

Davison TS, Vagner C, Kaghad M, Ayed A, Caput D, Arrowsmith CH. "p73 and p63 are homotetramers capable of weak heterotypic interactions with each other but not with p53." *J Biol Chem*., vol:274, pp:18709-18714, 1999

De Laurenzi V, Raschella G, Barcaroli D, Annicchiarico-Petruzzelli M, Ranalli M, Catani MV, Tanno B, Costanzo A, Levrero M, Melino G. "Induction of neuronal differentiation by p73 in a neuroblastoma cell line." *J Biol Chem*., vol:275, pp:15226-15231, 2000

DeCaprio, J.A., Ludlow, J.W., Figge, J., Shew, J.Y., Huang, C.M., Lee, W.H., Marsilio, E., Paucha, E., and Livingston, D.M. "SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene". *Cell* 54: 275-283, 1988.

DeGregori, J., Leone, G., Miron, A., Jakoi, L. & Nevins, J. R. "Distinct roles for E2F proteins in cell growth control and apoptosis". *Proc. Natl Acad. Sci*. USA 94, 7245-7250, 1997.

DeGregori, J., Leone, G., Ohtani, K., Miron, A. & Nevins, J. R. "E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity". *Genes Dev.* 9, 2873-2887, 1995.

Di Como CJ, Gaiddon C, Prives C. "p73 function is inhibited by tumor-derived p53 mutants in mammalian cells." *Mol Cell Biol*., vol: 19, pp: 1438-1449, 1999

Dobbelstein M, Wienzek S, Konig C, Roth J. “Inactivation of the p53-homologue p73 by the mdm2-oncoprotein.” *Oncogene*., vol:18, pp:2101-2106, 1999

Dominguez G, Silva JM, Silva J, Garcia JM, Sanchez A, Navarro A, Gallego I, Provencio M, Espana P, Bonilla F. “Wild type p73 over-expression and high-grade malignancy in breast cancer.”, *Breast Cancer Res Treat*., vol:66, pp:183-190, 2001

Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R., and Goff, S.P. “The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest”. *Cell* 79: 119-130, 1994.

Durfee, T., Mancini, M.A., Jones, D., Elledge, S.J., and Lee, W.H. “The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing”. *J. Cell. Biol*. 127: 609-622, 1994.

Dyson, N. et al. “Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRb-associated E2F-1”. *J. Virol*. 67, 7641-7647, 1993.

Dyson, N. “The regulation of E2F by pRb-family proteins”. *Genes D*ev. 12, 2245-2262, 1998.

Dyson, N., Howley, P.M., Munger, K., and Harlow, E. “The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product”. *Science* 243: 934-937, 1989.

El-Naggar AK, Lai S, Clayman GL, Mims B, Lippman SM, Coombes M, Luna MA, Lozano G. “p73 gene alterations and expression in primary oral and laryngeal squamous carcinomas.” *Carcinogenesis*, vol:22, pp:729-735, 2001

Eng, C., Li, F.P., Abramson, D.H., Ellsworth, R.M., Wong, F.L., Goldman, M.B., Seddon, J., Tarbell, N., and Boice, J.J. “Mortality from second tumors among long-term survivors of retinoblastoma”. *J. Natl. Cancer* Inst. 85, 1993.

Ewen, M.E., Faha, B., Harlow, E., and Livingston, D.M. “Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins”. *Science* 255: 85-87, 1992.

Ezhevsky, S.A., Nagahara, H., Vocero, A.A., Gius, D.R., Wei, M.C., and Dowdy, S.F. “Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:CDK4/6 complexes results in active pRb”. *Proc. Natl. Acad. Sci*. 94: 10699-10704, 1997.

Fang L, Lee SW, Aaronson SA. “Comparative analysis of p73 and p53 regulation and effector functions.” *J Cell Biol*., vol:147, pp:823-830, 1999

Faridoni-Laurens L, Bosq J, Janot F, Vayssade M, Le Bihan ML, Kaghad M, Caput D, Benard J, Ahomadegbe JC. “P73 expression in basal layers of head and neck squamous epithelium: a role in differentiation and carcinogenesis in concert with p53 and p63?” *Oncogene*, vol:20, pp:5302-5312, 2001

Ferreira, R., Magnaghi-Jaulin, L., Robin, P., Harel-Bellan, A., and Trouche, D. “The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase”. Pr*oc. Natl. Acad. Sci*. 95: 10493-10498, 1998.

Fillippovich I, et al. “Transactivation Deficient p73alpha inhibits apoptosis and competes with p53”. *Oncogene*, Vol: 20, 514-522, 2001.

Flemington, E.K., Speck, S.H., and Kaelin, W.G., Jr. “E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product”. *Proc. Natl. Acad. Sci*. 90: 6914-6918, 1993.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., and Dryja, T.P. “A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma”. *Nature* 323: 643-646, 1986.

Fukushima K, Ueno Y, Yamagiwa Y, Yamakawa M, Iwasaki T, Ishii M, Toyota T, Shimosegawa T. “Correlation between p21(waf1) and p16(INK4a) expression in hepatocellular carcinoma.” *Hepatol Res.,* vol:20, pp:52-67, 2001

Fung, Y.K., Murphree, A.L., T'Ang, A., Qian, J., Hinrichs, S.H., and Benedict, W.F. “Structural evidence for the authenticity of the human retinoblastoma gene”. *Science* 236: 1657-1661, 1987.

Gaiddon C, Lokshin M, Ahn J, Zhang T, Prives C. “A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain.” *Mol Cell Biol.,* vol:21, pp:1874-1887, 2001

Gaubatz, S., Wood, J. G. & Livingston, D. M. “Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F-6”. *Proc. Natl Acad. Sci*. USA 95, 9190-9195, 1998.

Giaccia AJ, Kastan MB. “The complexity of p53 modulation: emerging patterns from divergent signals.” *Genes Dev*., vol:12, pp:2973-2983, 1998

Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin WG Jr, Levrero M, Wang JY. “The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage.” *Nature*, vol:399, pp:806-809, 1999

Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. “Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation”. *Cell* 72: 309-324, 1993.

Han S, Semba S, Abe T, Makino N, Furukawa T, Fukushige S, Takahashi H, Sakurada A, Sato M, Shiiba K, Matsuno S, Nimura Y, Nakagawara A, Horii A. “Infrequent somatic mutations of the p73 gene in various human cancers.” *Eur J Surg Oncol*., vol:25, pp:194-198, 1999

Hanahan D. “Studies on transformation of Escherichia coli with plasmids”. *J Mol Biol*. Vol:166(4) pages: 557-80, 1983

Hannon, G.J., Demetrick, D., and Beach, D.. “Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins”. Genes & Dev. 7: 2378-2391, 1993

Harbour, J.W., Lai, S.L., Whang-Peng, J., Gazdar, A.F., Minna, J.D., and Kaye, F.J. “Abnormalities in structure and expression of the human retinoblastoma gene in SCLC”. *Science* 241: 353-357, 1988.

Harbour, J.W., Luo, R.X., Dei Sante, A., Postigo, A.A., and Dean, D.C. “CDK phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1”. *Cell* 98: 859-869, 1999.

Helin, K. et al. “Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation”. *Genes Dev*. 7, 1850-1861, 1993.

Helin, K. “Regulation of cell proliferation by the E2F transcription factors”. *Curr. Opin. Genet. De*v. 8, 28-35, 1998.

Helin, K., Harlow, E., and Fattaey, A. “Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein”. *Mol. Cell. Biol*. 13: 6501-6508, 1993.

Herbst RA, Mommert S, Schubach J, Podewski EK, Kapp A, Weiss J. “Allelic loss at the p73 locus (1p36.33) is infrequent in malignant melanoma.” *Arch Dermatol Res*., vol:291, pp:362-364, 1999

Hijmans, E.M., Voorhoeve, P.M., Beijersbergen, R.L., van'tVeer, L.J., and Bernards, R. “E2F-5, a new E2F family member that interacts with p130 in vivo”. *Mol. Cell. Biol*. 15: 3082-3089, 1995.

Holstege, F.C., Jennings, E.G., Wyrick, J.J., Lee, T.I., Hengartner, C.J., Green, M.R., Golub, T.R., Lander, E.S., and Young, R.A. “Dissecting the regulatory circuitry of a eukaryotic genome”. *Cell* 95: 717-728, 1998.

Horowitz, J.M., Park, S.H., Bogenmann, E., Cheng, J.C., Yandell, D.W., Kaye, F.J., Minna, J.D., Dryja, T.P., and Weinberg, R.A. “Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells”. *Proc. Natl. Acad. Sci*. 87: 2775-2779, 1990.

Hsiao, K. M., McMahon, S. L. & Farnham, P. J. “Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter”. *Genes Dev. 8*, 1526-1537, 1994.

Hsieh, J.K., Chan, F.S., O'Connor, D.J., Mittnacht, S., Zhong, S., and Lu, X. “RB regulates the stability and the apoptotic function of p53 via MDM2”. *Mol. Cell* 3: 181-193, 1999.

Hsieh JK, Yap D, O'Connor DJ, Fogal V, Fallis L, Chan F, Zhong S, Lu X. “Novel function of the cyclin A binding site of E2F in regulating p53-induced apoptosis in response to DNA damage”. Mol Cell Biol Vol:22(1) pp:78-93, 2002.

Hsu IC, Tokiwa T, Bennett W, Metcalf RA, Welsh JA, Sun T, Harris CC. “p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines”. *Carcinogenesis*. Vol: 14(5) pp:987-92. 1993.

Huang, H.J., Yee, J.K., Shew, J.Y., Chen, P.L., Bookstein, R., Friedmann, T, Lee, E.Y., and Lee, W.H. “Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells”. *Science* 242: 1563-1566, 1988.

Huang, S., Shin, E., Sheppard, K.A., Chokroverty, L., Shan, B., Qian, Y.W., Lee, E.Y., and Yee, A.S. “The retinoblastoma protein region required for interaction with the E2F transcription factor includes the T/E1A binding and carboxy-terminal sequences”. *DNA Cell. Biol*. 11: 539-548, 1992.

Humbert, P. O. et al. “E2F3 is critical for normal cellular proliferation”. *Genes Dev.* 14, 690-703, 2000.

Iavarone, A. & Massague, J. “E2F and histone deacetylase mediate transforming growth factor-β repression of cdc25A during keratinocyte cell cycle arrest”. *Mol. Cell. Biol*. 19, 916-922, 1999.

Ichimiya S, Nimura Y, Kageyama H, Takada N, Sunahara M, Shishikura T, Nakamura Y, Sakiyama S, Seki N, Ohira M, Kaneko Y, McKeon F, Caput D, Nakagawara A. “Genetic analysis of p73 localized at chromosome 1p36.3 in primary neuroblastomas.” *Med Pediatr Oncol*., vol:36, pp:42-44, 2001

Ichimiya S, Nimura Y, Kageyama H, Takada N, Sunahara M, Shishikura T, Nakamura Y, Sakiyama S, Seki N, Ohira M, Kaneko Y, McKeon F, Caput D, Nakagawara A. “p73 at chromosome 1p36.3 is lost in advanced stage neuroblastoma but its mutation is infrequent.” *Oncogene* vol:18, pp:1061-1066, 1999

Ikeda, M. A., Jakoi, L. & Nevins, J. R. “A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation”. *Proc. Natl Acad. Sci*. USA 93, 3215-3220, 1996.

Inoue H, Nojima H, Okayama H. “ High efficiency Transformation of E.Coli with plasmids”. Gene, Vol: 96, 23-28, 1990

Irwin, M. et al. “Role for the p53 homologue p73 in E2F-1-induced apoptosis”. *Nature* 407, 645-648, 2000.

Irwin MS, Kaelin WG. “ p53 Family Update: p73 and p63 develop Their Own identities”. *Cell Growth and Differentiation*. Vol:12, 337-349, 2001

Ivey-Hoyle, M. et al. “Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F”. *Mol. Cell. Biol.* 13, 7802-7812, 1993.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and Weinberg, R.A. “Effects of an Rb mutation in the mouse”. *Nature* 359: 295-300, 1992.

Ji H, Stout LE, Zhang Q, Zhang R, Leung HT, Leung BS. “Absence of transforming growth factor-beta responsiveness in the tamoxifen growth-inhibited human breast cancer cell line CAMA-1”. *J Cell Biochem.* Vol:54(3) pp:332-42, 1994.

Johnson, D. G., Cress, W. D., Jakoi, L. & Nevins, J. R. “Oncogenic capacity of the E2F1 gene”. *Proc. Natl Acad. Sci*. USA 91, 12823-12827, 1994.

Johnson, D. G., Schwarz, J. K., Cress, W. D. & Nevins, J. R. “Expression of transcription factor E2F1 induces quiescent cells to enter S phase”. *Nature* 365, 349-352, 1993.

Jost CA, Marin MC, Kaelin WG Jr. “p73 is a simian [correction of human] p53-related protein that can induce apoptosis” *Nature*, vol:389, pp:191-194, 1997

Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, Caput D. “Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers.” *Cell*, vol:90, pp:809-819, 1997

Kang MJ, Park BJ, Byun DS, Park JI, Kim HJ, Park JH, Chi SG. “Loss of imprinting and elevated expression of wild-type p73 in human gastric adenocarcinoma.” *Clin Cancer Res*., vol:6, pp:1767-1771, 2000

Kartasheva NN, Contente A, Lenz-Stoppler C, Roth J, Dobbelstein M. “p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop”. *Oncogene*. Vol: 21(31) pp:4715, 2002.

Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H, Sakashita A, Said J, Tatsumi E, Koeffler HP. “Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation.” *Blood*, vol:94, pp:1113-1120, 1999

Kiess, M., Gill, R.M., and Hamel, P.A. “Expression and activity of the retinoblastoma protein (pRb)-family proteins, p107 and p130, during L6 myoblast differentiation”. *Cell Growth Differ*. 6: 1287-1298, 1995.

Kim, H.Y. and Cho, Y. “Structural similarity between the pocket region of retinoblastoma tumour suppressor and the cyclin-box”. *Nat. Struct. Biol*. 4: 390-395, 1997.

Kingston, R.E. and Narlikar, G.J. “ATP-dependent remodeling and acetylation as regulators of chromatin fluidity”. *Genes & Dev*. 13: 2339-2352, 1999.

Knudsen, E.S. and Wang, J.Y. “Differential regulation of retinoblastoma protein function by specific CDK phosphorylation sites”. *J. Biol. Chem*. 271: 8313-8320, 1996.

Knudsen, E.S. and Wang, J.Y. “Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation”. *Mol. Cell. Biol*. 17: 5771-5783, 1997.

Kong XT, Valentine VA, Rowe ST, Valentine MB, Ragsdale ST, Jones BG, Wilkinson DA, Brodeur GM, Cohn SL, Look AT. “Lack of homozygously inactivated p73 in single-copy MYCN primary neuroblastomas and neuroblastoma cell lines.” *Neoplasia*, vol:1, vol:80-89, 1999

Kornberg, R.D. and Lorch, Y. “Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome”. *Cell* 98: 285-294, 1999.

Kovalev S, Marchenko N, Swendeman S, LaQuaglia M, Moll UM. “Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines.” Cell Growth Differ., vol:9, pp:897-903, 1998

Kowalik, T. F., DeGregori, J., Leone, G., Jakoi, L. & Nevins, J. R. “E2F1-specific induction of apoptosis and p53 accumulation, which is blocked by Mdm2”. *Cell Growth Differ*. 9, 113-118, 1998.

Krek, W., Livingston, D. M. & Shirodkar, S. “Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members”. *Science* 262, 1557-1560, 1993.

Kroiss MM, Bosserhoff AK, Vogt T, Buettner R, Bogenrieder T, Landthaler M, Stolz W. "Loss of expression or mutations in the p73 tumour suppressor gene are not involved in the pathogenesis of malignant melanomas." *Melanoma Res*., vol:8, pp:504-509, 1998

Lacy, S. and Whyte, P. "Identification of a p130 domain mediating interactions with cyclin A/CDK 2 and cyclin E/CDK 2 complexes". *Oncogene* 14: 2395-4206, 1997.

Lai, A., Lee, J.M., Yang, W.M., DeCaprio, J.A., Kaelin, W.G., Jr., Seto, E., and Branton, P.E. "RBP1 recruits both histone deacetylase-dependent and -independent repression activities to retinoblastoma family proteins". Mol. *Cell. Biol*. 19: 6632-6641, 1999a.

Lai, A., Marcellus, R.C., Corbeil, H.B., and Branton, P.E. "RBP1 induces growth arrest by repression of E2F-dependent transcription". *Oncogene* 18: 2091-2100, 1999b.

Lam, E. W. & Watson, R. J. "An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription". *EMBO J.* 12, 2705-2713, 1993.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H, and Bradley, A. "Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis". *Nature* 359: 288-294, 1992

Lee, E.Y., To, H., Shew, J.Y., Bookstein, R., Scully, P., and Lee, W.H. "Inactivation of the retinoblastoma susceptibility gene in human breast cancers". *Science* 241: 218-221, 1988.

Lee, J.O., Russo, A.A., and Pavletich, N.P. "Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7". *Nature* 391: 859-865, 1998.

Lee, M.H., Williams, B.O. Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and Jacks, T. "Targeted disruption of p107: Functional overlap between p107 and Rb". *Genes & Dev*. 10: 1621-1632, 1996.

Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y., and Lee, E.Y. "Human retinoblastoma susceptibility gene: cloning, identification, and sequence". *Science* 235: 1394-1399, 1987.

Lees, J. A. et al. "The retinoblastoma protein binds to a family of E2F transcription factors". *Mol. Cell. Biol*. 13, 7813-7825, 1993.

Leone, G. et al. "E2F3 activity is regulated during the cell cycle and is required for the induction of S phase". *Genes Dev*. 12, 2120-2130, 1998.

Leone, G. et al. "Myc requires distinct E2F activities to induce S phase and apoptosis". *Mol. Cell* 8, 105-113, 2001.

Levrero M, De laurenzi V, Costanzo A, Sabatini S, Gong J, Wang, JYJ, Melino, G. "The p53/p63/p73 Family of Transcription Factors; Overlapping and Distinct Functions". *Journal of Cell Science*. 113, 1661-1670, 2000.

Lin, W. C., Lin, F. T. & Nevins, J. R. "Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation". *Genes Dev.* **15**, 1833-1844, 2001.

Lissy, N. A., Davis, P. K., Irwin, M., Kaelin, W. G. & Dowdy, S. F. "A common E2F-1 and p73 pathway mediates cell death induced by TCR activation". *Nature* 407, 642-645, 2000.

Liu W, Mai M, Yokomizo A, Qian C, Tindall DJ, Smith DI, Thibodeau SN. "Differential expression and allelotyping of the p73 gene in neuroblastoma." *Int J Oncol*., vol:16, pp:181-185, 2000

Lomas J, Bello MJ, Arjona D, Gonzalez-Gomez P, Alonso ME, de Campos JM, VaqueroJ, Ruiz-Barnes P, Sarasa JL, Casartelli C, Rey JA. "Analysis of p73 gene in meningiomas with deletion at 1p" *Cancer Genet Cytogenet*., vol:129, pp:88-91, 2001

Lorch, Y., Zhang, M., and Kornberg, R.D. "Histone octamer transfer by a chromatin-remodeling complex". *Cell* 96: 389-392, 1999.

Ludlow, J.W., DeCaprio, J.A., Huang, C.M., Lee, W.H., Paucha, E., and Livingston, D.M. "SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family". *Cell* 56: 57-65, 1989.

Lukas, J., Petersen, B. O., Holm, K., Bartek, J. & Helin, K. "Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression". *Mol. Cell. Biol*. 16, 1047-1057, 1996.

Lundberg, A.S. and Weinberg, R.A. "Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-CDK complexes". *Mol. Cell. Biol*. 18: 753-761, 1998.

Luo, R.X., Postigo, A.A., and Dean, D.C. "Rb interacts with histone deacetylase to repress transcription". *Cell* 92: 463-473, 1998.

Magnaghi, J.L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le, V.J., Troalen, F., Trouche, D., and Harel, B.A. "Retinoblastoma protein represses transcription by recruiting a histone deacetylase". *Nature* 391: 601-605, 1998.

Mai M, Huang H, Reed C, Qian C, Smith JS, Alderete B, Jenkins R, Smith DI, Liu W. "Genomic organization and mutation analysis of p73 in

oligodendrogliomas with chromosome 1 p-arm deletions." *Genomics*, vol:51, pp:359-363, 1998

Mai M, Yokomizo A, Qian C, Yang P, Tindall DJ, Smith DI, Liu W. "Activation of p73 silent allele in lung cancer." *Cancer Res.,* vol:58, pp:2347-2349, 1998

Mann, D. J. & Jones, N. C. "E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest". *Curr. Biol*. 6, 474-483, 1996.

Martinez-Balbas, M.A., Bauer, U.M., Nielsen, S.J., Brehm, A., and Kouzarides, T. "Regulation of E2F1 activity by acetylation". *EMBO J.* 19: 662-671, 2000.

Maser, R. S. et al. "Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis". *Mol. Cell. Biol*. 21, 6006-6016, 2001.

Meloni, A.R., Smith, E.J., and Nevins, J.R. "A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor". *Proc. Natl. Acad*. Sci. 96: 9574-9579, 1999.

Meng, R. D., Phillips, P. & El-Deiry, W. S. "p53-independent increase in E2F-1 expression enhances the cytotoxic effects of etoposide and of adriamycin". *Int. J. Oncol.* **14**, 5-14, 1999.

Mihara M, Nimura Y, Ichimiya S, Sakiyama S, Kajikawa S, Adachi W, Amano J, Nakagawara A. "Absence of mutation of the p73 gene localized at chromosome 1p36.3 in hepatocellular carcinoma." *Br J Cancer*, vol:79, pp:164-167, 1999

Moberg, K., Starz, M. A. & Lees, J. A. "E2F-4 switches from p130 to p107 and pRb in response to cell-cycle re-entry". *Mol. Cell. Biol*. 16, 1436-1449, 1996.

Moll, A.C., Imhof, S.M., Bouter, L.M., and Tan, K.E. "Second primary tumors in patients with retinoblastoma". A review of the literature. *Ophthalmic Gene*t. 18: 27-34, 1997.

Momoi H, Okabe H, Kamikawa T, Satoh S, Ikai I, Yamamoto M, Nakagawara A, Shimahara Y, Yamaoka Y, Fukumoto M. "Comprehensive allelotyping of human intrahepatic cholangiocarcinoma." *Clin Cancer Res*., vol: 9, pp:2648-2655, 2001

Mulligan, G. & Jacks, T. "The retinoblastoma gene family: cousins with overlapping interests". *Trends Genet.* 14, 223-229, 1998.

Nakano K, Balint E, Ashcroft M, Vousden KH. "A ribonucleotide reductase gene is a transcriptional target of p53 and p73." *Oncogene*, vol:19, pp:4283-4289, 2000

Nevins, J.R. “Toward an understanding of the functional complexity of the E2F and retinoblastoma families”. *Cell Growth Differ*. 9: 585-593, 1998.

Ng SW, Yiu GK, Liu Y, Huang LW, Palnati M, Jun SH, Berkowitz RS, Mok SC. “Analysis of p73 in human borderline and invasive ovarian tumor.” *Oncogene*, vol:19, pp:1885-1890, 2000

Nimura Y, Mihara M, Ichimiya S, Sakiyama S, Seki N, Ohira M, Nomura N, Fujimori M, Adachi W, Amano J, He M, Ping YM, Nakagawara A. “p73, a gene related to p53, is not mutated in esophageal carcinomas”. *Int J Cance*r, vol:78, pp:437-440, 1998

Nomoto S, Haruki N, Kondo M, Konishi H, Takahashi T, Takahashi T, Takahashi T. “Search for mutations and examination of allelic expression imbalance of the p73 gene at 1p36.33 in human lung cancers.” *Cancer Res*., vol:58, pp:1380-1383, 1998

Nozaki M, Tada M, Kashiwazaki H, Hamou MF, Diserens AC, Shinohe Y, Sawamura Y, Iwasaki Y, de Tribolet N, Hegi ME. “p73 is not mutated in meningiomas as determined with a functional yeast assay but p73 expression increases with tumor grade.” *Brain Pathol*., vol:11, pp:296-305, 2001

Ongkeko WM, Wang XQ, Siu WY, Lau AW, Yamashita K, Harris AL, Cox LS, Poon RY. “MDM2 and MDMX bind and stabilize the p53-related protein p73.” *Curr Biol*., vol:9, pp:829-832, 1999

O'Nions J, Brooks LA, Sullivan A, Bell A, Dunne B, Rozycka M, Reddy A, Tidy JA, Evans D, Farrell PJ, Evans A, Gasco M, Gusterson B, Crook T. “p73 is over-expressed in vulval cancer principally as the Delta 2 isoform.” *Br J Cancer*, vol:85, pp:1551-1556, 2001

Peng CY, Tsai SL, Yeh CT, Hung SP, Chen MF, Chen TC, Chu CM, Liaw YF. “Genetic alternations of p73 are infrequent but may occur in early stage hepatocellular carcinoma.” *Anticancer Res.,* vol:20(3A), pp:1487-1492, 2000

Peters MA, Janer M, Kolb S, Jarvik GP, Ostrander EA, Stanford JL. “Germline mutations in the p73 gene do not predispose to familial prostate-brain cancer.” *Prostate*, vol: 48, pp:292-296, 2001

Phillips, A. C., Ernst, M. K., Bates, S., Rice, N. R. & Vousden, K. H. “E2F-1 potentiates cell death by blocking antiapoptotic signaling pathways”. *Mol. Cell 4*, 771-781, 1999.

Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD. “An anti-apoptotic role for the p53 family member, p73, during developmental neuron death.” *Science,* volo:289, pp:304-306 , 2000

Puisieux A, Galvin K, Troalen F, Bressac B, Marcais C, Galun E, Ponchel F, Yakicier C, Ji J, Ozturk M. "Retinoblastoma and p53 tumor suppressor genes in human hepatoma cell lines". *FASEB J.* Vol: 7(14) pp:1407-13, 1993.

Qin, X. Q., Livingston, D. M., Kaelin, W. G. Jr & Adams, P. D. "Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl Acad. Sci*. USA 91, 10918-10922, 1994.

Qin, X.Q., Chittenden, T., Livingston, D.M., and Kaelin, W.G., Jr. "Identification of a growth suppression domain within the retinoblastoma gene product". *Genes & Dev*. 6: 953-964, 1992.

Robanus-Maandag, E., Dekker, M., van der Valk, M., Carrozza, M.L., Jeanny, J.C., Dannenberg, J.H., Berns, A., and te Riele, H. "p107 is a suppressor of retinoblastoma development in pRb-deficient mice". *Genes & Dev*. 12: 1599-1609, 1998.

Ryan BM, McManus R, Daly JS, Carton E, Keeling PW, Reynolds JV, Kelleher D. "503 A common p73 polymorphism is associated with a reduced incidence of oesophageal carcinoma." *Br J Cancer*, vol: 85, pp:1499-1503, 2001

Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R.A. "E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle". *Proc. Natl. Acad. Sci*. 92: 2403-2407, 1995.

Sayan AE, Sayan BS, Findikli N, Ozturk M. "Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells". *Oncogene*. Vol:20, 5111-5117, 2001

Sayan BS, Ince A, Sayan AE, Ozturk M. " NAPO as novel marker of apoptosis". *Journal of Cell Biology*, Volume 155-5, 2001

Schaeper, U., Subramanian, T., Lim, L., Boyd, J.M., and Chinnadurai, G. "Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif". *J. Biol. Chem*. 273: 8549-8552, 1998.

Schittek B, Sauer B, Garbe C. "Lack of p73 mutations and late occurrence of p73 allelic deletions in melanoma tissues and cell lines." *Int J Cancer*, vol:82, pp:583-586, 1999

Schnitzler, G., Sif, S., and Kingston, R.E. "Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state". *Cell* 94: 17-27, 1998.

Schwartz DI, Lindor NM, Walsh-Vockley C, Roche PC, Mai M, Smith DI, Liu W, Couch FJ. "p73 mutations are not detected in sporadic and hereditary breast cancer." *Breast Cancer Res Treat.*, vol:58, pp:25-29, 1999

Schwarz, J. K. et al. “Expression of the E2F1 transcription factor overcomes type-Beta transforming growth factor-mediated growth suppression”. *Proc. Natl Acad. Sci.* USA 92, 483-487, 1995.

Sellers, W.R., Rodgers, J.W., and Kaelin, W.G., Jr. “A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites*”. Proc. Natl. Acad*. Sci. 92: 11544-11548, 1995.

Serrano M. “The INK4a/ARF locus in murine tumorigenesis.” *Carcinogenesis,* vol:21, pp:865-869, 2000

Shan L, Yang Q, Nakamura Y, Nakamura M, Miyauchi A, Tsujimoto M, Nakatani Y, Wakasa K, Mori I, Kakudo K. “Frequent loss of heterozygosity at 1p36.3 and p73 abnormality in parathyroid adenomas.” *Mod Pathol*., vol: 14, pp:273-278, 2001

Shan, B. & Lee, W. H. “Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis”. *Mol. Cell. Biol.* 14, 8166-8173, 1994.

Sherr CJ and Weber JD. “The ARF/p53 pathway.” *Curr Opin Genet Dev*., vol: 10, pp:94-99, 2000

Sherr CJ, Roberts JM. “CDK inhibitors: positive and negative regulators of G1-phase progression.” *Genes Dev*., vol:13, pp:1501-1512, 1999

Sherr, C.J. “Cancer cell cycles”. *Science* 274: 1672-1677, 1996.

Shew JY, Lin BT, Chen PL, Tseng BY, Yang-Feng TL, Lee WH. “C-terminal truncation of the retinoblastoma gene product leads to functional inactivation”. *Proc Natl Acad Sci U S A.* Vol: 87(1) pp: 6-10. 1990

Shin, E.K., Shin, A., Paulding, C., Schaffhausen, B., and Yee, A.S. “Multiple change in E2F function and regulation occur upon muscle differentiation”. *Mol. Cell. Biol*. 15: 2252-2262, 1995.

Shishikura T, Ichimiya S, Ozaki T, Nimura Y, Kageyama H, Nakamura Y, Sakiyama S, Miyauchi M, Yamamoto N, Suzuki M, Nakajima N, Nakagawara A. “Mutational analysis of the p73 gene in human breast cancers.” *Int J Cancer*, vol:84, pp:321-325, 1999

Singh, P., Wong, S. H. & Hong, W. “Over-expression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation”. *EMBO J.* 13, 3329-3338, 1994.

Soussi T, Beroud C. “Assessing TP53 status in human tumours to evaluate clinical outcome.” *Nature Rev. Cancer*, vol:1, pp:233-240, 2001

Spillare EA, Okamoto A, Hagiwara K, Demetrick DJ, Serrano M, Beach D, Harris CC. “Suppression of growth in vitro and tumorigenicity in vivo of human

carcinoma cell lines by transfected $p16^{INK4}$". *Mol Carcinog*. Vol: 16(1) pp: 53-60. 1996

Stanelle J, Stiewe T, Theseling CC, Peter M, Putzer BM. "Gene expression changes in response to E2F1 activation." *Nucleic Acids Res.*, vol:30, pp:1859-1867, 2002

Starostik, P., Chow, K.N., and Dean, D.C. "Transcriptional repression and growth suppression by the p107 pocket protein". *Mol. Cell. Biol.* 16: 3606-3614, 1996.

Sterner, J.M., Dew, K.S., Musahl, C., Kornbluth, S., and Horowitz, J.M. "Negative regulation of DNA replication by the retinoblastoma protein is mediated by its association with Mcm7". *Mol. Cell. Biol.* 18: 2748-2757, 1998.

Sterner, J.M., Murata, Y., Kim, H.G., Kennett, S.B., Templeton, D.J., and Horowitz, J.M. "Detection of a novel cell cycle-regulated kinase activity that associates with the amino terminus of the retinoblastoma protein in G2/M phases". *J. Biol. Chem*. 270: 9281-9288, 1995.

Stiewe T and Putzer BM. "Role of the p53-homologue p73 in E2F1-induced apoptosis" *Nat Genet.,* vol: 26, pp: 464-469, 2000

Stiewe T, Putzer BM. "Role of p73 in malignancy: tumor suppressor or oncogene?" *Cell Death Differ*., vol" 9, pp: 237-245, 2002

Stiewe T, Theseling CC, Putzer BM. "Transactivation-deficient Delta TA-p73 Inhibits p53 by Direct Competition for DNA Binding. IMPLICATIONS FOR TUMORIGENESIS." *J Biol Chem*., vol:277, pp:14177-14185, 2002

Stirewalt DL, Clurman B, Appelbaum FR, Willman CL, Radich JP. "p73 mutations and expression in adult de novo acute myelogenous leukemia." *Leukemia*, vol:13, pp:985-990, 1999

Strano S, Munarriz E, Rossi M, Cristofanelli B, Shaul Y, Castagnoli L, Levine AJ, Sacchi A, Cesareni G, Oren M, Blandino G. "Physical and functional interaction between p53 mutants and different isoforms of p73." *J Biol Chem.*, vol:275, pp:29503-29512, 2000

Strobeck, M.W., Knudsen, K.E., Fribourg, A.F., DiCristofaro, M.F., Weissman, B.E., Imbalzano, A.N., and Knudsen, E.S. "BRG-1 is required for RB-mediated cell cycle arrest". *Proc. Natl. Acad. Sci*. 97: 7748-7753, 2000.

Sunahara M, Ichimiya S, Nimura Y, Takada N, Sakiyama S, Sato Y, Todo S, Adachi W, Amano J, Nakagawara A. "Mutational analysis of the p73 gene localized at chromosome 1p36.3 in colorectal carcinomas." *Int J Oncol*., vol:13, pp:319-323, 1998

Takahashi H, Ichimiya S, Nimura Y, Watanabe M, Furusato M, Wakui S, Yatani R, Aizawa S, Nakagawara A. “Mutation, allelotyping, and transcription analyzes of the p73 gene in prostatic carcinoma.” *Cancer Res*., vol:58, pp:2076-2077, 1998

Takahashi, Y., Rayman, J. B. & Dynlacht, B. D. “Analysis of promoter binding by the E2F and pRb families in vivo: distinct E2F proteins mediate activation and repression*”. Genes Dev*. 14, 804-816, 2000.

T'Ang, A., Varley, J.M., Chakraborty, S., Murphree, A.L., and Fung, Y.K. “Structural rearrangement of the retinoblastoma gene in human breast carcinoma”. *Science* 242: 263-266, 1988.

Templeton, D.J., Park, S.H., Lanier, L., and Weinberg, R.A. “Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering”. *Proc. Natl. Acad. Sci*. 88: 3033-3037, 1991.

Trimarchi, J. M. et al. “E2F-6, a member of the E2F family that can behave as a transcriptional repressor”. *Proc. Natl Acad. Sci. USA* 95, 2850-2855, 1998.

Trouche, D., Cook, A., and Kouzarides, T. “The CBP co-activator stimulates E2F1/DP1 activity”. *Nucleic Acids Res*. 24: 4139-4145, 1996.

Trouche, D., Le, C.C., Muchardt, C., Yaniv, M., and Kouzarides, T. “Rb and hbrm cooperate to repress the activation functions of E2F1”. *Proc. Natl. Acad. Sci.* 94: 11268-11273, 1997.

Tsai, K. Y. et al. “Mutation of E2F-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos”. *Mol. Cell* 2, 293-304, 1998.

Tsao H, Zhang X, Majewski P, Haluska FG. “Mutational and expression analysis of the p73 gene in melanoma cell lines.” *Cancer Res,* vol:59, pp:172-174, 1999

Tsujimoto T, Mochizuchi S, Iwadate Y, Namba H, Nagai M, Kawamoto T, Sunahara M, Yamaura A, Nakagawara A, Sakiyama S, Tagawa M. “The p73 gene is not mutated in oligodendrogliomas which frequently have a deleted region at chromosome 1p36.3.” *Anticancer Res*., vol:20, pp:2495-2497, 2000

Tyler, J.K. and Kadonaga, J.T. “The "dark side" of chromatin remodeling: Repressive effects on transcription”. *Cell* 99: 443-446, 1999.

Ueda Y, Hijikata M, Takagi S, Chiba T, Shimotohno K. “New p73 variants with altered C-terminal structures have varied transcriptional activities.” *Oncogene*, vol:18, pp:4993-4998, 1999

Ueda Y, Hijikata M, Takagi S, Chiba T, Shimotohno K. "Transcriptional activities of p73 splicing variants are regulated by inter-variant association." *Biochem J*., vol:356, pp:859-866, 2001

Vairo, G., Livingston, D. M. & Ginsberg, D. "Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members". *Genes Dev*. 9, 869-881, 1995.

Van Gele M, Kaghad M, Leonard JH, Van Roy N, Naeyaert JM, Geerts ML, Van Belle S, Cocquyt V, Bridge J, Sciot R, De Wolf-Peeters C, De Paepe A, Caput D, Speleman F. "Mutation analysis of P73 and TP53 in Merkel cell carcinoma." *Br J Cancer*, vol:82, pp:823-826, 2000

Wang J, Helin K, Jin P, Nadal-Ginard B. Inhibition of in vitro myogenic differentiation by cellular transcription factor E2F1. *Cell Growth Differ.* Vol: **6**: 1299-1306, 1995

Verona, R. et al. "E2F activity is regulated by cell cycle-dependent changes in subcellular localization". *Mol. Cell. Biol*. 17, 7268-7282, 1997.

Vigo, E. et al. "CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase". *Mol. Cell. Biol*. 19, 6379-6395, 1999.

Weber A, Bellmann U, Bootz F, Wittekind C, Tannapfel A. "Expression of p53 and its homologues in primary and recurrent squamous cell carcinomas of the head and neck." *Int J Cancer*, vol:99, pp: 22-28, 2002.

Weinberg, R.A. "The retinoblastoma protein and cell cycle control". *Cell* 81: 323-330, 1995.

Weintraub, S.J., Chow, K.N., Luo, R.X., Zhang, S.H., He, S., and Dean, D.C. "Mechanism of active transcriptional repression by the retinoblastoma protein". *Nature* 375: 812-815, 1995.

Welch, P.J. and Wang, J.Y. A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. *Cell* 75: 779-790, 1993.

Wells, J., Boyd, K. E., Fry, C. J., Bartley, S. M. & Farnham, P. J. "Target gene specificity of E2F and pocket protein family members in living cells". *Mol. Cell. Biol*. 20, 5797-5807, 2000.

Whitaker, L.L., Su, H., Baskaran, R., Knudsen, E.S., and Wang, J.Y.J. "Growth suppression by an E2F-binding-defective retinoblastoma protein (RB): Contribution from the RB C pocket". *Mol. Cell. Biol*. 18: 4032-4042, 1998.

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A., and Harlow, E. "Association between an oncogene and an

anti-oncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product". *Nature* 334: 124-129, 1988.

Wolffe, A.P. and Hayes, J.J. "Chromatin disruption and modification". *Nucleic Acids Res.* 27: 711-720, 1999.

Wu X, Levine AJ. "p53 and E2F-1 cooperate to mediate apoptosis". *Proc Natl Acad Sci USA* Vol: **91** pp:3602-3606, 1994.

Wu, C. L., Zukerberg, L. R., Ngwu, C., Harlow, E. & Lees, J. A. "In vivo association of E2F and DP family proteins". *Mol. Cell. Biol.* 15, 2536-2546, 1995.

Wu, L. et al. "The E2F1-3 transcription factors are essential for cellular proliferation". *Nature* 414, 457-462, 2001.

Xiao, Z.X., Chen, J., Levine, A.J., Modjtahedi, N., Xing, J., Sellers, W.R., and Livingston, D.M. "Interaction between the retinoblastoma protein and the oncoprotein Mdm2". *Nature* 375: 694-698, 1995.

Xu, G., Livingston, D. M. & Krek, W. "Multiple members of the E2F transcription factor family are the products of oncogenes". *Proc. Natl Acad. Sci. USA* 92, 1357-1361, 1995.

Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D. "p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours." *Nature*, vol:404, pp:99-103, 2000

Yee, A.S., Shih, H.H., and Tevosian, S.G. "New perspectives on retinoblastoma family functions in differentiation". *Front. Biosci.* 3: D532-D547, 1998.

Yokomizo A, Mai M, Bostwick DG, Tindall DJ, Qian J, Cheng L, Jenkins RB, Smith DI, Liu W. "Mutation and expression analysis of the p73 gene in prostate cancer." *Prostate*, vol:39, pp:94-100, 1999

Yokomizo A, Mai M, Tindall DJ, Cheng L, Bostwick DG, Naito S, Smith DI, Liu W. "Over-expression of the wild type p73 gene in human bladder cancer." *Oncogene*, vol:18, pp:1629-1633, 1999

Yokozaki H, Shitara Y, Fujimoto J, Hiyama T, Yasui W, Tahara E. "Alterations of p73 preferentially occur in gastric adenocarcinomas with foveolar epithelial phenotype." *Int J Cancer*, vol:83, pp:192-196, 1999

Yoshikawa H, Nagashima M, Khan MA, McMenamin MG, Hagiwara K, Harris CC. "Mutational analysis of p73 and p53 in human cancer cell lines." *Oncogene*, vol:18, pp:3415-3421, 1999

Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R, Kufe D. "p73 is regulated by tyrosine kinase c-Abl in

the apoptotic response to DNA damage." *Nature*, vol:399, pp:814-817, 1999

Zaika AI, Kovalev S, Marchenko ND, Moll UM. "Over-expression of the wild type p73 gene in breast cancer tissues and cell lines." *Cancer Res.*, vol:59, pp:3257-3263, 1999

Zaika A, Irwin M, Sansome C, Moll UM. "Oncogenes Induce and Activate Endogenous p73 Protein". *The Journal Of Biological Chemistry*. Vol:276, 11310-11316, 2001

Zamanian, M. and La, T.N. "Transcriptional repression by the Rb-related protein p107". *Mol. Biol. Cell* 4: 389-396, 1993.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., and Dean, D.C. "Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF". *Cell* 101: 79-89, 2000.

Zhu, J. W. et al. "E2F1 and E2F2 determine thresholds for antigen-induced T-cell proliferation and suppress tumorigenesis". *Mol. Cell. Biol*. 21, 8547-8564, 2001.

Zhu, L., Harlow, E., and Dynlacht, B.D. "p107 uses a p21CIP1-related domain to bind cyclin/CDK2 and regulate interactions with E2F". *Genes & Dev*. 9: 1740-1752, 1995b.

Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. "Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein". *Genes & Dev*. 7: 1111-1125, 1993.

Ziebold, U., Reza, T., Caron, A. & Lees, J. A. "E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos". *Genes Dev*. 15, 386-391, 2001.